

“STUDIES ON THE LEAVES OF *Solanum melongena* var.*melongena*  
AND ITS PROMISING ACE INHIBITION FOR VARIOUS THERAPEUTIC  
APPROACHES

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# INTRODUCTION

## Plants as sources for medicine

It was well known to ancient world that plants are a rich source of a variety of chemicals with nutritive and therapeutic properties. Herbs are seen as potential medicine for a variety of diseases often view to supercede the pharmacological efficacy of allopathic drugs. The striking increase in the use of herbals in both developing and developed countries is due to their natural origin and minimal/no side effects.<sup>1</sup>

Plants have been one of the important sources of medicines since the dawn of human civilization. 1/3<sup>rd</sup> of the world's population treat themselves with traditional medicines. A continued search for medicinal plants during the last several centuries has given rise to a long list of plants which are of great use in the treatment of diseases and for promoting health.<sup>2</sup>

It is estimated that there are 2,50,000 to 5,00,000 species of plants on earth. An estimated 35,000 to 70,000 are being used for therapeutic purpose. Thousands of years human have relied on natural product as the primary source of medicines. Plants form the basis for traditional medicine system and continue to play an essential

role in health care. It has been estimated by WHO that approximately 80% of world's inhabitants rely mainly on traditional medicine for their primary health care.<sup>1</sup>

### **Medicinal plants in India**

India is perhaps the largest producer of medicinal herbs and is rightly called the **“Botanical Garden of the World.”** Since independence in 1947, India has made tremendous progress in agrotechnology, process technology, standardization, quality control, research and development etc. In India the earliest mention of the use of medicinal plants is to be found in the Rigveda (4500-1600 BC). Even now 75% of the Indian population depends on this indigenous system for relief.

India has 15,000-18,000 species of flowering plants, 2500 algae, 23,000 fungi, 1,600 types of lichen, 1800 varieties of bryophytes and an estimated 30 million types of micro-organisms. Of these about 15,000-20,000 plants have good medicinal value. However only about 7,000-7,500 are used for their medicinal values by traditional communities. The Siddha system of medicines uses about 600, Ayurveda 700, Unani 700, and modern medicines about 30. During the last two decades over 3,000 plants have been screened in India for their biological activities. About 130 pure chemical substances extracted from some 100 species of higher plants and are used as medicines throughout the world.<sup>3</sup>

## **Herbal medicine for healing and health**

Herbal medicine is older than any other type of health care. Every culture has taken advantage of herbs and their benefits. Man's knowledge of herbs and their medicinal uses advanced overtime. Herbal medicine continues to influence the medicines of today. Presently, approximately 25% of the prescription drugs are sold in the United States are plant based. Of the 119 plant-derived pharmaceutical drugs, as many as 74% are used in the same ways the plants were used by the natives. Plants gathered from locations such as the rain forests are being studied for their possible medicinal values by various pharmaceutical companies.

Conditions such as high blood pressure, asthma, pain, and heart diseases are often treated today with commercial medicines containing plant-based substances. Herbs serve as therapeutic agents as well as important raw materials for the manufacture of traditional modern medicine. People in the United States are continually gaining interest in herbs because of an increasing number of success stories. One example is the use of St.John's Wort to treat forms of depression to avoid using Prozac which produces unwanted side effects.<sup>4</sup>

## **Alternative system of medicine**

### **AYURVEDA**

The origin of Ayurveda has been lost in prehistoric antiquity (2500-500 BC). The word Ayurveda derived from 'Ayur' meaning life and 'Veda' meaning science. Ayurveda is said to be Upaveda (part) of Atharvana Veda, this describes 341 plants and plant products for use in medicine. The Sushruta Samhita (600 BC) which has special emphasis on surgery, described 395 medicinal plants, 57 drugs of animal origin, 64 minerals and metals as therapeutic agents. The ayurvedic preparations are complex mixtures including plant and animal derived products, minerals, and metals.

Modern methods of chemistry, biochemistry, and clinical research are being used to find out the utility of a particular Ayurveda drug and find out its active chemical constituents. This is being done in research institutions which are wholly modern in their outlook. Through the research carried out on Ayurveda herbs and medicine, all the methods and appliances used belong to modern biochemistry and clinical research.<sup>5</sup>

### **Siddha system of medicine**

Sage Agasthya is considered the Patron saint of the Siddha system of medicine. He authored various texts in Siddha medicine which are available even today. The Siddha system of medicine is the



oldest and was derived from the vegetable kingdom. Siddhi generally refers to the Ashtama Siddhi i.e. the eight supernatural powers. Those who attained or achieved these powers are known as the siddhars. The siddhars' knowledge of Iatro-chemistry, minerals, metals and plants was stupendous. This was successfully used by them from time immemorial. Herbs are used for trituration or calcination. The metals and minerals used in Siddha medicine are completely in a detoxified state as per the method known as “Shodana.”<sup>6</sup>

### **Unani system of medicine**

Unani medicine originated from ancient Greece. In 460BC the Greek philosopher and father of modern medicine, Bukharath (Hippocrates) who freed medicine from the clutches of superstition and laid the foundation of Unani medicine. Unani medicine plays a vital role when the individual experiences the humoral imbalance. The correct diet and digestion can bring back the humoral imbalance. Diet therapy aims at treating certain ailments by administration of specific diets. Its main emphasis is on diagnosis of the disease through pulse, urine, stools, etc. It has laid down six essential prerequisites for the prevention

of disease. So diet and the use of naturally occurring medicines (herbal) are very important in the treatment of illness.<sup>7</sup>

## **PHYTOPHARMACEUTICALS**

Phytopharmaceuticals form an important part of herbal drugs industry and so called allopathic system of medicine has also recognized their importance. Many of the drugs used in their system e.g. sex and other hormones, anti-cancers, and cardio-vascular drugs are derived from herbal sources. The demonstration of the presence of natural products, viz. polyphenols, alkaloids, flavanoids, and other secondary metabolites in medicinal plants will provide a scientific validation for the popular use of them and serve as a guide, which may help in the selection of the plants with therapeutic effect.<sup>5</sup>

### **Contributions of Pharmacognosy to modern medicine**

The advances in Pharmacognosy is associated with simultaneous advancement in the areas of organic chemistry, biochemistry, biosynthesis, and modern methods and techniques of analysis like paper, thin layer, gas and HPTLC. Thus a wide variety of active principles isolated from different plants were established to possess various pharmacological activities.<sup>8</sup>

<b>PLANT PRODUCTS</b>	<b>THERAPEUTIC USES</b>
Steroids	Useful for the manufacture of oral contraceptives and other steroidal hormones.
Ephedrine	For asthma and other respiratory symptoms.
Digoxin, Digitoxin (Digitalis) used as medicine since 1775	Cardiac stimulant
Morphine(Opium poppy), used since 1804	Narcotic Analgesic
Crude gum guggal (Commifora mukul)	Lowering the serum cholesterol level

In the recent years, there is noticeable increase in the percentage of chronic diseases (cancer, HIV, heart diseases, diabetes) affecting mankind. In that list oral diseases also having major contribution. A recent review discussed possible etiological associations between periodontitis (the progressive destruction of the supporting structures of the teeth which is triggered by bacterial plaque) and cardiovascular disease. Periodontal inflammation facilitates the entrance of bacteria into the bloodstream, especially after chewing food or cleaning teeth. Either direct effects from the bacteremia or secondary effects from the inflammation which their presence may trigger could lead to thrombus formation and/or the development of atherosclerotic lesions. This

suggests a potentially valuable role for phytotherapy in assisting with the management of dental plaque.

### **Oral infections and systemic diseases**

Oral diseases, including tooth decay, gingivitis and periodontitis, are the most prevalent afflictions of humankind. Furthermore, it has been suggested in recent years that oral bacteria are associated with many systemic diseases such as pneumonia, cardiovascular disease, premature, and low birth weight babies.

The health of gums, teeth and mouth are very important to your overall health, since the research shows the connection between poor oral health and systemic disease such as diabetes in people of all ages and respiratory diseases particularly among elderly people.

**Gum disease:** Gum disease is a common ailment facing many adults. It is an inflammation of the gums, bones and tissues that surround and support teeth. Gum disease can be difficult to recognize in its early stages as it develops slowly without any real pain. Gums are important with maintaining the health of your mouth.

Healthy gums are:

- Pink; not red in color.
- Firm.
- Free from inflammation or swelling.
- Resistant to bleeding during brushing and/or flossing.

**Gingivitis:** Gingivitis is the first stage of gum disease. Gingivitis is the inflammation of the gum tissue.

Gingivitis is characterized by:

- Red and swollen (puffy) gums
- Pain in the gum area
- Blood on your toothbrush or floss
- Persistent bad breath

**Oral cancer:** Oral cancer is any abnormal growth and spread of cells occurring in the mouth cavity including the: <sup>9</sup>

- Lips;
- Inside of the lips and cheeks;
- Tongue;
- Gums;
- Floor of the mouth;

## Dental plaque

For more than a century, dental plaque has been a major target of chemoprophylaxis and with regard to periodontal diseases, of chemotherapy. It can be defined as the soft deposits that form the biofilm adhering to the tooth surface or other hard surfaces in the oral cavity. The existence of micro-organisms as the polyspecies consortium known as “Dental plaque” has profound implications in the etiology of caries, gingivitis, and periodontal diseases.

Dental plaque in other words an oral biofilm comprising a multi-species community that forms on the surfaces of the oral cavity. A cavity is caused when the bacteria living in the plaque react with sugars from the food or drink we eat, resulting in an acid. This acid then attacks the surface of the tooth. It can be painful if the cavity is not stopped and it progresses inside the tooth structure.

Although the oral microbial flora is quite diverse and complex, two species of mutans streptococci in the biofilm, *Streptococcus mutans* and *Streptococcus sobrinus* have generally been regarded as the primary etiologic agents of dental caries in humans.<sup>10, 11,12</sup>

## ACTORS INFLUENCING PLAQUE FORMATION

- Rapid transport of dietary sugars: the **sugar phosphotransferase uptake system** is a high affinity process. *Mutans streptococci* possess more than one sugar transport system.
- Rapid rates of glycolysis (acidogenicity): can result in a terminal pH of below 4.5 in only a few minutes.
- Tolerance of, and growth at, low pH (aciduricity): the growth of many of the bacteria found on sound enamel.
- Extracellular polysaccharide synthesis (EPS): these polymers help make up the plaque matrix. Glucosyltransferases (GTF's) convert sucrose to soluble and insoluble glucans, that help consolidate bacterial attachment; ***Strep. mutans*** also produces a specific highly insoluble polymer (**mutan**). Fructosyltransferases (FTF's) convert sucrose to **fructans**; these polymers are labile and can be used by plaque bacteria as an energy source.
- Intracellular polysaccharide synthesis (IPS): can be used during starvation conditions and catabolised to acid when dietary sugars are not available.<sup>12</sup>



### **Health hazards due to dental plaque**

- Dental decay is a disease that can damage the tooth's structure.

Decay starts by damaging the tooth's protective coating, also known as enamel, causing a hole (cavity) to develop in your tooth. If the cavity is left untreated, it can get bigger and, besides causing pain, this could lead to the loss of a tooth.

- Gingivitis begins with the build-up of plaque on your teeth. The bacteria in plaque mix with sugar from the foods that you eat to produce acids that can attack the surfaces of the teeth and gums.

- Plaque can harden into tartar which can help contribute to a more serious form of gum disease called periodontal disease. Periodontal disease severely affects the bone and gums that support and keep teeth in their place. Periodontal disease can lead to weakening the gums and ultimately to tooth loss. It is not possible to reverse the damage caused by periodontal disease, but it is possible to prevent it in the first place.

- Plaques and tartar is deposited mainly in the gaps between the teeth and gum. This will provide shelter for the food debris and bacteria causing bad breath.

- Colonization of aerobic respiratory pathogens dental plaques may be an important reservoir for pneumonia in institutionalized elders.<sup>13</sup>

### **Herbal treatment for plaque**

It can be used either as a primary treatment or a supplementary treatment for a certain condition such as having dental plaques. Herbal treatment for dental plaque has been proven by users to be very effective. Herbal treatment, especially herbal treatment for plaque, has no known harmful side effects. Instead of using the raw materials, herbs are now studied and extracted and are used to make herbal medicines used for herbal treatment.

The most common herbs used in the treatment of plaque are as follows:

**Pomegranate:** The fruit has been scientifically proven that the fruits of *Punica granatum* is effective against dental plaque microorganisms.

**Cranberry:** With the scientific name *Vaccinium macrocarpon*, Cranberry has also been known to be effective against the formation of dental plaque. **Sage:** Scientifically known as *Salvia officinalis*, Sage can act as an anti-bacterial agent against plaques. This can be prepared by adding two teaspoons of its powder extract to 2 cups of boiling water. Gargle and spit. Popular plants which are fashioned into chewing sticks

include neem and arak (**Salvadora persica**). Aqueous extracts of **neem** revealed that alteration of bacterial properties and ability of Streptococci to colonize tooth surface are inhibited. Azadirachta indica stem are used as chewing sticks. African members of the Meliaceae such as the **Crabwood** or Monkey Cola Tree (Carapa procera) and are also used as chewing-sticks .<sup>13</sup>

### **Reason for the selecting *Wrightia tinctoria* (Roxb.) R.Br.**

Several plants species are known to have helped in cure, treatment of periodontal diseases, particularly in alleviation of tooth aches. In the literature, several plants have been referred to as commonly used plants for dental care. In recent years, on account of adverse effects of synthetic drugs, attempts have been made upon the potential of phytochemicals for the prevention and treatment of dental plaque diseases.

Keeping these in view, the ethnomedical information, the leaves of ***Wrightia tinctoria***, Family: Apocynaceae chewed by the tribal peoples for the relief of tooth ache.<sup>14</sup> Despite the traditional use of ***W.tinctoria*** leaf as a relief for tooth ache, its effect on oral diseases particularly on biofilm related diseases such as dental caries remains unknown. Moreover preliminary literature survey revealed the presence of tryptanthrin as one of the constituents in the leaves of ***W.tinctoria*** which was proved to have antibacterial activity.<sup>15,16</sup> Based on these facts we planned to study the effect of leaves of ***W.tinctoria*** on the biofilm formation.

# REVIEW OF LITERATURE

## WHOLE PLANT

### Ethnomedical Information

**Chopra R.N.**, (1955) In Indian indigenous medicine ***W.tinctoria*** is found to be useful in dysentery.<sup>17</sup>

**Joshi P.**, (1993) Extracts of ***W.tinctoria*** used as remedies for snake and scorpion stings by the tribals of Rajasthan.<sup>18</sup>

**Thangadurai D.**, (1998) It is used as an antidote for snake and scorpion bite by the jungle tribals of Tirunelveli District of TN.<sup>19</sup>

**Behera L.M, Sen S.K.**, (2007) It was reported that ***W.tinctoria*** is used in the treatment of gynecological disorders.<sup>20</sup>

**Ignacimuthu S.** reported that the plant is useful in treating female reproductive disorders by the traditional folklore practitioners of Western Ghats of TN uses.<sup>21</sup>

**Ravishankar T.** reported that the plant is useful in treating mumps and as lactagogue.<sup>22</sup>

(**Dr. Dukes** phytochemical and ethnobotanical data base) It is used for tooth ache, aphrodisiac, bilious, carminative, dropsy, hypertension, stomachic, and as vermifuge.<sup>23</sup>

## Pharmacognostical Studies

**Narayana Iyer K.** (1960) The external morphology, inflorescence, flowers, fruits, and seeds have been discussed.<sup>24</sup>

**Prakash N.A,** et al., (1999) A semi root parasite and slow growing tree namely Sandal (*Santalum album* L.) and its growth measurement under different forest host plants including *W.tinctoria* to increase the biomass production of sandal have been reported.<sup>25</sup>

**Thippeswamy G.** et al., (2003) Ten plant extracts including *W.tinctoria* was used to treat the seeds of some oil seed crop species like sesame, groundnut, castor and niger. In case of sesame, castor, and niger the plant extract showed better inhibitory activity against fungi. Additionally the plant ext treatment resulted in enhanced emergence and germination. The seedling symptoms such as browning, wilt leaf-rot and damping off were also controlled by plant extract treatment.<sup>26</sup>

**Suresh D.R,** et al., (2004) Cultivation by vegetative propagation were discussed.<sup>27</sup>

**Anil Kumar A.S,** et al., (2007) Biofencing of medicinal trees and its importance including *W.tinctoria* in humid tropics region have been discussed.<sup>28</sup>

The life cycle, climatic factors, traditional uses, propagation methods, and horticulture of the plant have been reported.<sup>29</sup>

**Raju C.V.** member of Etikoppaka Vana Samrakshana Samiti reported that 67,000 ***W.tinctoria*** tree saplings were planted during 1998-2001.<sup>30</sup>

### **Phytochemical studies**

**Sethi PD**, (1970) Alkaloid constituents of the plant were separated by TLC.<sup>31</sup>

### **Pharmacological Screening**

#### **Antidiarrheal activity**

(**Rajendran VM**, et al.,(1979)Antidiarrheal activity of the aqueous extract has been evaluated in mouse. (100mg of extract/animal p.o). The extract prevented diarrhea completely in case of Myrobalan and Epsom salt.<sup>32</sup>

(**Mustahasan J**, (1993) Four Unani herbal drugs including Lisanul Asafir (***W.tinctoria***) were exemplified for its therapeutic use by the Unani physicians of 19<sup>th</sup> Century AD. This pharmacological studies on these drugs indicated the empirical rationale behind inclusion of these drugs in Unani system of medicine.<sup>33</sup>

(**Ahmad S**, et al., (1994) Haemostatic activity of the Unani formulation containing ***W.tinctoria*** has been standardized for its therapeutic use and to check adulteration.<sup>34</sup>

(**Chandrashekhar VM**, et al., (2004) Hepatoprotective activity of ***W.tinctoria*** was studied in rats.(against Ccl4 induced liver damage).<sup>35</sup>

## **Microbiological Studies**

### **Antifungal activity**

(**Krishnamurthy J.R**, et al., (2000) Antipityrosporum activity of herbal drug combination containing ***W.tinctoria*** and Hisbiscus rosasinensis was tested invitro against isolates of Pityrosporum ovale recovered from dandruff. The drug combination exhibited a fungicidal activity at a concentration ranging between 500 to 1000 mcg/ml.<sup>36</sup>

(**Krishnamoorthy J.R**, et al., (2006) Dano, a polyherbal antidandruff oil prepared from ***W.tinctoria***, Cassia alata, and bitter fraction of neem. Microbiological (against P.ovale and Candida albicans) and clinical tests were carried out. Eight days of Dano use had reduced the scaling from severe to mild to traces to nil. The antifungal activity coupled with keratinocyte



proliferation inhibition of the *W.tinctoria* in Dano makes the oil very effective in the treatment of dandruff.<sup>37</sup>

(Alluri V, et al.,) The plant was screened for cytotoxicity using Brine Shrimp Lethality test with Podophylotoxin as standard.

(LC<sub>50</sub> >5000µg/ml).<sup>38</sup>

### **Antibacterial activity**

**Raman Dang** et al., (2005) Antibacterial activity of petroleum ether, chloroform, methanol, and aqueous extracts of the plant was studied by diffusion method using *Staphylococcus aureus*

(gram+ve) *Escherichia coli* (gram-ve) as test organisms.

Results suggest that plants extracts are effective against both gram(+) and (-) organisms.<sup>39</sup>

**TABLE NO – 1**  
**ANTIBACTERIAL ACTIVITY OF VARIOUS EXTRACTS OF**  
***W.tinctoria***

<b>Extract</b>	<b>Zone of Inhibition</b>	<b>Organism</b>
Petroleum ether extract	10 to 20 mm	S.aureus and E.coli
Chloroform extract	24 mm	S.aureus
Aqueous extract	14 mm	E.coli

## **LEAF**

### **Ethnomedical Information**

**Sebastian MK, Bhandari MM**, (1984) Latex from the leaf used as styptic. 40

**Reddy M.B**, et al.,(1989) Ethnobotanical survey was conducted during 1982-87 at Anantapur district of Andrapradesh was reported that 92 useful plant drugs were used by the herbalists. In that list leaf paste of the plant applied externally to treat head lice.<sup>41</sup>

**Siddiqui, MB** et al.,(1990) Leaves and bark are grounded with water to make a fine paste and applied locally for snakebite. Decoction of bark and leaf is taken orally for snakebite.<sup>42</sup>

**Nagaraju.N, Rao KN,** (1990) Tribal and nontribal inhabitants of Rayalaseema of AP uses 131 useful plants for various ailments have been presented. Decoction of the stem bark and leaf is useful in treating piles.<sup>43</sup>

**Tiwari VJ, Padhye MD,** (1993) It was reported that latex from the leaf taken orally to treat asthma and bronchitis.<sup>44</sup>

**Selvanayahgam ZE** et al., (1994) Leaves applied externally and used as antivenin. Decoction of the bark and leaf taken orally used as an antidote.<sup>45</sup>

**Selvanayahgam ZE** et al., (1995) Irula tribes, traditional medical practitioners, and local informants in Chengalpattu district of TN apply externally the latex from the leaf for snakebite.<sup>46</sup>

**Reddy K.N,** et al., (2006) Ethnomedical survey around Eastern Ghats of AP revealed that 84 species belonging to 72 genera and 41 families were used as remedy for respiratory disorders by the rural and forest ethnic people. They use the extract of crushed leaves of ***W.tinctoria***, Piper longum, Allium to cure asthma.<sup>47</sup>

**Reddy K.N,** et al., (2006) Erukulas , Koyas in Eastern Ghats of AP apply leaf juice of “Tedla Paala” as nasal drops to get free breathe.<sup>47</sup>

**Chiranjibi Pattanaik** et al., 2006) Milky latex applied externally on base of teeth for toothache.<sup>48</sup>

**Ganesan S,** (2007) The leaf paste mixed with neem is applied for eczema by the valaiyan group of Alagarkovil Hills.<sup>49</sup>

**Kottaimuthu R.** (2008) Valaiyans of Karandamalai, Dindigul district apply leaf paste of Vetpali on aching teeth to relief pain from tooth ache.<sup>14</sup>

**Tomar R. et al.,** (2008) Wrightin, a stable serine protease isolated from the latex is used in food and biotechnological industries.<sup>50</sup>

US Patent No-5858372 documented for the pharmaceutical preparation for psoriasis containing latex of ***W.tinctoria***, PEG, and urea.<sup>51</sup>

### **Pharmacognostical Studies**

□ No pharmacognostical studies were reported in the leaves.

### **Phytochemical Studies**

- **N.Rageswara Rao** (1966) Chemical examination of the leaf extract were studied which revealed the presence of  $\beta$  amyrin, ursolic acid, and ursolic acid derivatives.<sup>52</sup>
- **Daniel.M** et al., (1978) It was reported that the fresh leaves of *Wrightia* contains four hydroxybenzoic acid, 2(OH) 6 methoxy benzoic acid, ferulic acid, O glycoflavones, quercetin, and benzenoid.<sup>53</sup>

### Standardization of 777 oil

- **Alam M.** et al., (1994) Analytical values of 777 oil prepared from fresh whole, fresh cut and dry leaves of *W.tinctoria* have been reported. The oil prepared by varying ingredients did not show any difference in chromatography. The variation of quality of leaf and oil indicated high acid number when leaves were double the weight of oil.<sup>54</sup>
- **Saraswathy.A** (1999) 777 oil, siddha preparation, its in depth study was made using HPTLC finger prints and comparative study with various extracts of leaves with the objective of laying down Pharmacopoeial standards.<sup>55</sup>

- **Muruganandan A.V**, et al., (1998) Members of the genus *Wrightia* have been investigated.(HPTLC, HPLC, and spectroscopic studies)

#### MAJOR CONSTITUENTS OF MEMBERS OF GENUS WRIGHTIA

<b>W.tinctoria</b>	<b>W.tomentosa</b>	<b>W.coccinea</b>
Indigotin, Indirubin, Tryptanthrin, isatin, anthranillate, and rutin	Same as <i>W.tinctoria</i>	Anthranillate and Rutin

In this Indigotin is found in fresh leaves, but indirubin formed during drying process, presumably caused by the hydrolytic enzyme system and oxidation. Steady increase of Indigotin and Indirubin from August to November, but isatin and anthranillate in the month of December and January, at the expense of Indigotin-indirubin.<sup>56</sup>

- **George.V** et al., (2003) Sisairosp an oily antipsoriatic preparation formulated from the leaf extract of ***W.tinctoria***, *Azadirachta indica*, and root extract *Hemidesmus indicus*. The chemistry of the oil has been discussed.<sup>57</sup>

- **Tomar.R** (2008) ***Wrightin***, a protease (monomer enzyme) from the latex of the leaf is purified by cation exchange chromatography and its biochemical properties were studied. *Wrightin* hydrolyzes casein, azoalbumin, and hemoglobin, but failed to hydrolyze trypsin like enzymes.<sup>51</sup>

**TABLE NO – 2**  
**BIOCHEMICAL PROPERTIES OF WRIGHTIN FROM *W.tinctoria***

Molecular mass	57.9 KDa
Iso electric point	6
Extinction Coefficient	36.4
O.D seen	At pH 7.5-10, temp at 70 <sup>0</sup> C

### **Pharmacological Studies**

#### **Anti-inflammatory activity**

- **Ghosh D** et al., (1985) It was reported that 777 oil exhibited significant anti-inflammatory activity in rats (1.5, 3, 6 mg/kg p.o). In addition graded analgesic effect was recorded in chemical writhing test as well as on thermal stimulus.<sup>58</sup>

#### **Psoriasis treatment**

- **Rao K.K, Shetty** et al., (1989) Twenty two cases of non-specific dermatitis were treated with 777 oil and significant response was seen within six weeks of treatment.<sup>59</sup>
- **Rekha.N** (2000) The importance of samshodhana therapy using Arogyavardhini vati and Arauvadhadhi kashayam (p.o) before the palliative treatment using Kutaja taila (containing ***W.tinctoria***) in case of psoriasis were analyzed by clinical trials on 22 cases of psoriasis.<sup>60</sup>

- **George V** (2003) Clinical trials on Saisirosp, an antipsoriatic oil preparation was reported.<sup>57</sup>
- **Venkataranganna M.V, Gopumadhavan,** (1998) An experimental histological evaluation on reversal parakeratosis based on mouse tail test was conducted on ***W.tinctoria***. Application of ***W.tinctoria*** on tails showed 90% reversal of parakeratosis when compared to betamethasone cream.<sup>61</sup>

**Anxiolytic and Antidepressant of *W.tinctoria*(Effect of brain monoamine and its metabolite)**

- **Muruganandan A.V,** et al., (1998) Administration of methanolic extract modulates the brain monoamines (serotonin, 5HIAA, NE, MHPG, DA) and its metabolites. This was studied in rats by injecting (25 and 50 mg/kg, i.p). After 30 minutes of injection the brain monoamines and its metabolites were assayed in five different brain regions (hypothalamus, hippocampus, striatum, pons medulla and frontal cortex). The modulation of neurotransmitters explains the anxiolytic and antidepressant effects of the extract.<sup>56</sup>



## Biological Studies

### Antibacterial Activity

- **George M, Pandalai K.M,** (1949) Ethanolic and hot water extracts of leaf were tested for antibacterial activity by disc diffusion method. *Staphylococcus aureus* and *E.coli* were employed. It was reported that hot water extract is found to be active against *E.coli*, but inactive to *S.aureus*. On the other case ethanolic extract was found to be inactive for both.<sup>62</sup>
- **Kannan P,** et al., (2006) Methanolic and ethanolic extracts showed antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* (MIC 0.5, 0.25 mg/ml). Hexane extract showed antifungal activity against dermatophytic fungi.<sup>67</sup>

### Psoriasis treatment

- **Muzaffar A,** et al., (1985) In humans, the administration of lipid fraction is found to be active in psoriasis treatment.<sup>63</sup>

### ACE Inhibition

- **Nyman U,** et al., (1998) Angiotensin converting enzyme inhibition was tested on aqueous, ethanolic, and acetone extracts of shade dried leaf and twig at 0.33 mg/ml concentration. Acetone extract

showed moderate activity than water and ethanolic extract which had weak activity.<sup>64</sup>

### **Antimicrobial Activity**

- **Anbuganapathi G**, et al., (2002) Chloroform, ethanol, and aqueous extracts of ***W.tinctoria*** were investigated for antimicrobial activity against various microorganisms. Among the three, the order of inhibition was chloroform>ethanol>aqueous.<sup>65</sup>

### **Antifungal Activity**

- **Vijayakumar R**, et al., (2006) ***W.tinctoria*** leaf extracts and seed oil showed significant antifungal property as they progressively inhibited the growth of *M.furfur* on Sabouraud's dextrose agar medium.<sup>66</sup>

### **FLOWER**

#### **Ethnomedical Information**

- **Selvanayagam Z.E.** (1995) Flower juice given orally to human adults for snake bite.<sup>45</sup>

### **Pharmacognostical Studies**

- **Jain S, Goyal S.L**, (1996) ***W.tinctoria*** have extra floral nectaries on the adaxial surface of the petiole and also associated with the shoot apex.<sup>68</sup>
- **Solomon Raju A.J**, et al., (2005) Pollination mechanism, pollinators, seed dispersal, and sexual system of the flowers has been studied.<sup>69</sup>

## Phytochemical Studies

- **Sethuraman V, et al., (1984).** Crude extract on fractionation yields yellow residue from Et<sub>2</sub>O fraction which was characterized as *quercetin* by its (melting point, spectral studies, m.p of its acetate). The residue from EtOAc subjected to paper chromatography (15% OHAC) a band corresponding to R<sub>f</sub> value 0.40 was cut and eluted with aqueous alcohol. From the eluate *rutin* was obtained, identified through physical, and chemical means and by comparing with authentic sample of *Rutin* obtained from *Strepbulus asper*.<sup>70</sup>

## Pharmacological Studies

### Anti-inflammatory activity

- **Sethuraman V. et al., (1984)** Crude extract of the flower showed anti-inflammatory activity in rats (30, 60, 120 mg/kg B.W). Carageenan induced rat paw edema significantly reduced in dose dependent manner(std-phenyl butazone). The ED<sub>50</sub> works out to 92 mg/kg. The presence of quercetin 3-O rhamnoglycoside may

be responsible for the anti-inflammatory activity.<sup>70</sup>

## **FRUITS**

### **Ethnomedical information**

- Fruit juice reported to possess anticoagulant effect.<sup>71</sup>

### **Phytochemical Studies**

- **Rao MN**, et al.,(1966) Presence of  $\alpha$  *amyrin*, *oleanolic* acid, and  $\beta$  *sitosterol* has been reported.<sup>72</sup>

### **Microbiological Studies**

- **Simonsen HT**, et al., (2001) Ethanolic extract of the dried fruit found to be active against *Plasmodium falciparum*. The extract was found to be equivocal and the IC<sub>50</sub> is 160 mcg/ml.<sup>73</sup>

## **SEED**

### **Ethnomedical information**

- **Kapoor SL, Kapoor LD**, (1980) Seeds given orally used as aphrodisiac.<sup>74</sup>
- **Nyman U** et al., (1998) Shade dried seeds of ***W.tinctoria*** given orally used as cardiotonic.<sup>75</sup>
- **Chellaiah Muthu**, et al., (2006) It was reported that seeds taken orally for indigestion by the traditional healers of Kancheepuram.<sup>76</sup>

## Pharmacognostical Studies

- **Jolly C.I, Mechery N.R**, (1996) Bitterness value of the seeds of *Holarrhena antidysentrica* and ***W.tinctoria*** was determined which showed seeds of ***W.tinctoria*** found to be less bitter than prior.<sup>77</sup>
- **Vasundhara M. et al.**, studied that growth regulator GA3 at 100-500 ppm level recorded significant 50% germination in the seeds of ***W.tinctoria***.<sup>78</sup>
- **Chauhan M.G, Patel Raj P**, (2008) Pharmacognosy and phytochemistry of seeds are compared with *H.antidysentrica* seeds. ***W.tinctoria*** seeds are not bitter, contains prismatic crystals, lignified papillose outgrowth. TLC separation did not show any spots of alkaloid, whereas *H.antidysentrica* seeds are highly bitter, contain rosette crystals, unlignified papillose outgrowth and TLC shows resolved spots of alkaloids.<sup>79</sup>

## Phytochemical Studies

- **Parihar, Dutt**, (1946) Seeds yield 30.49% of fixed oil.<sup>80</sup>
- **Smolenski SJ**, et al., (1974) Presence of alkaloid in the seeds have been reported.<sup>81</sup>

- **Fashih Ahmad**, et al., (1986) Structure of isoricinoleic acid and its moieties in the seeds were identified by chromatography and mass spectrophotometry.<sup>82</sup>
- **Akihisa T.** et al.,(1988) 14  $\alpha$  methylzymosterol was isolated from the unsaponifiable lipid of seeds.<sup>83</sup>
- **Ishtiaque Ahmad**, (2008) Seeds contain larger proportions of isoricinoleic acid (9-OH cis 12-octa decenoic acid). Acyl lipids which are identified by chromatography and mass spectroscopy. (triisoricinoleoyl glycerol, diisoricinoleoyl glycerol). Phospho and glycolipids contain only smaller proportions of isoricinoleoyl moieties.<sup>84</sup>

### **Pharmacological Studies**

- Nyman U, et al., (1998) Aqueous, ethanolic, and acetone extracts of seed were screened for ACE inhibition.(0.33 mg/ml)<sup>75</sup>

### **Microbiological Studies**

#### **Antifungal Activity**

- **Harish S**, et al., (2004) Seed extract at 10% exhibited antifungal activity against mycelia growth and spore germination of *H.oryzae*, a major constraint of rice production.<sup>85</sup>

### **POD**

### **Ethnomedical Information**

- **Ramachandra P**, et al., (1992) Decoction of the dried bark applied externally to treat psoriasis.<sup>86</sup>

### Phytochemical studies

- **Rangaswami S, Rao E.V**, (1960) Chemical examination of the powdered pods were carried out.  **$\alpha$  amyirin** is present in methanol soluble fraction of hexane extract. When sparingly soluble methanolic portion is chromatographed  **$\beta$  sitosterol** (m.p 134-136) by comparing it with that of standard.<sup>87</sup>
- **Ramachandra P**, et al.,(1992) **Cycloartenone**, **cycloenalenol**,  **$\alpha$  amyirin**,  **$\beta$  amyirin**,  **$\beta$  sitosterol**, **oleanolic acid** and **ursolic acid** were isolated from immature pods.<sup>86</sup>
- **Ramchandra P**, et al.(1993) From the methanolic extract of immature seed pod a new terpene namely Wrightial was isolated. By spectral and by chemical correlation, the structure of the Wrightial was established.<sup>88</sup>

Wrightial - 0.01%

$\beta$  amyirin - 0.15%

Cycloartenone - 0.02%

Cycloencalenol - 0.03%

## **BARK**

### **Ethno medical Information**

- **Joshi MC**, et al., (1980) Decoction of the dried stem bark given orally for abdominal pain.<sup>89</sup>
- **Singh V.P**, et al., (1980) Dried bark in India used to treat hemorrhages.<sup>90</sup>
- **Shah GL, Gopal GV**, 1985) Tribals of North Gujarat uses the fresh bark to treat wounds.<sup>91</sup>
- **Reddy M.B**, et al., (1989) Decoction prepared from the mixture of 40 g of stem bark, *Tinospora cordifolia*, root bark of *Echinops echinatus* and the seeds of *Nigella hispanica* (15-20g) given orally to treat fever.<sup>41</sup>
- **Vaidyaratnam Varier P.S**, (1996) Latex of the bark and unripe fruits are used by the hill tribes for coagulating and solidifying milk.<sup>93</sup>
- **Singh VK**, et al., (1996) In Bahraich Dt of UP, fresh stem bark given orally to female for contraception.<sup>92</sup>
- **Mudaliar KSM**, Siddha Materia Medica, (1998) Bark and seeds used as carminative, digestive, depurative, and anthelmintic.<sup>94</sup>
- One teaspoon of bark powder prepared from *Aegle marmelis*, Kurchi, and *W.tinctoria* were taken orally to treat dysentery.<sup>95</sup>



## Pharmacognostical Studies

- **Chopra et al.**, (1958) The bark is commonly used as adulterant of an important antidiarrheal drug *Holarrhena antidysenterica* another Apocynaceae plant.<sup>96</sup>
- **Reddy et al.**, (1999) Morphological, anatomical, and phytochemical characters of ***W.tinctoria*** bark have been presented and compared with that of *Holarrhena antidysenterica*.<sup>97</sup>

## Phytochemical Studies

- **Veerapur V.** et al., (2005) Dried bark contains triterpene namely  $\alpha$  and  $\beta$  amyrin.<sup>98</sup>
- **Bigoniya P.** et al., (2008) Qualitative phytochemical investigation revealed the presence of steroidal saponin, alkaloid, reducing sugar, tannins, flavanoids, and absence of glycoside.<sup>99</sup>

## Pharmacological Studies

### Antinociceptive Activity

- **Reddy Y.S.R** et al., (2002) Ethyl acetate, acetone, and methanolic extracts (200 mg/kg, i.g) screened for antinociceptive activity on mice. Extracts showed significant activity (Std: Acetyl salicylic acid.) The presence of terpenoids, flavanoids, and

steroids may be responsible for the observed pharmacological activity.<sup>100</sup>

- **Bigoniya** et al., (2006) Ethanolic extract of the bark showed immunomodulatory and good antiulcer activity in rats.<sup>99</sup>

### **Anti-inflammatory Activity**

- **Bigoniya** et al., (2006) Significant anti-inflammatory activity was reported in rats using hydroalcoholic extract .(Suppressant at 500 mg/kg, highly significant at 1000 mg/kg).<sup>99</sup>

### **Analgesic Effect**

- **Bigoniya** et al., (2006) Analgesic effect of hydroalcoholic extract was studied in Wistar rats by Eddy's hot plate method. Pain inhibition percentage of extract is (101.12, for std Morphine -496.42%).<sup>99</sup>

### **Pregnancy Prevention**

- **Keshri G**, et al., (2008) Ethanolic extract of the stembark inhibited pregnancy in 100% of rats (250 mg/kg, postcoitum). The contraceptive action of the ethanolic extract might be due to its estrogen agonistic activity.<sup>101</sup>

## Microbiological Studies

- **Ahmad I**, et al., (1998) Alcoholic extract of bark at 20% concentration possessed weak activity against *E.coli*, *Shigella Flexneri*, *S.Sonnei*, *S.boydii*, *Vibrio cholerae*.<sup>102</sup>

## WOOD

### Ethnomedical Information

- **Dastur J.F**, (1956) The wood is very valuable and extensively used for carving, making combs, toys, yokes, cups, plates, and pen-holders.<sup>103</sup>

## ROOTBARK

### Ethnomedical Information

- **Sudarsanam G**, et al., (1995) Rootbark is useful in treating snake and scorpion sting bite.<sup>104</sup>

## **VARIOUS METHODS TO EVALUATE PLAQUE**

1. **Ju Hee Song** et al., (2007) Invitro plaques were formed on saliva coated Hydroxyapatite discs WXD (10x2 mm). the discs were placed in 24-well polystyrene cell culture plates with BHI broth and *Streptococcus mutans*. The plates were incubated for 48 hours. The drug treated biofilms were dispersed using sonicator, diluted, and plated on BHI agar, to study the effects of extract on biofilm inhibition.<sup>11</sup>
  
2. **Seemann R.** et al., (2005) Microbial based caries model was developed to study the caries formation. Specimens were fixed on a rotating mount within a reaction chamber hermetically surrounded by a sterilized glove box. A cariogenic environment was obtained by inoculation with *S.mutans* with continuous supply of sucrose, trypticase soy broth, and artificial saliva. Test specimens were infected with *S.mutans* and were incubated for another 14 days. Demineralizations were evaluated by using confocal laser scanning microscopy.<sup>105</sup>

3. **Tanzer J.M**, et al., (1977) In vitro plaques were formed on No:20 Nichrome wires. The wires were fixed to culture tubes containing *S.mutans* and fluid thioglycolate medium. Preformed plaques were dipped in testing agents for various durations and frequencies. Plaques were judged to have been killed by the cessation of culture acid production and by failure of 24-h post treatment plaque samples to grow when plated on appropriate agar media.<sup>106</sup>

4. **Victor D. Warner** et al., (1975) 8(OH) Quinolone and its derivatives, potent antibacterial agents tested for their plaque inhibitory activity. Dental plaque were formed on sterilized extracted human teeth which are pretreated with test solutions in DMSO then they are immersed in trypticase broth containing *S.mutans* and 5% sucrose. The teeth are suspended in test tubes on orthodontic wire. Growth ratings of the plaque and the percentage inhibition of the test compound were calculated.<sup>107</sup>

5. **Yanti** et al., (2008) Antibiofilm activity of Macelignan, isolated from nutmeg was experimented. Biofilms of primary colonizer bacterium were prepared in commercially available presterilized, polystyrene, flat bottom 96-well microtitre plate. The plates were conditioned with 200 µl mucin as artificial saliva, Biofilms were initially grown in BHI broth supplemented with 5% sucrose. The plates were added with test and std solutions and incubated at 37°C. Adherent bacteria in the plates after washing were stained with crystal violet and the amount of crystal violet stain in the detaining solution was measured with a tunable microplate reader at 596 nm.<sup>108</sup>

6. **Sissons C.H, (1997) Biofilm technologies:** Biofilm technologies like non-biofilm culture systems, microbial biofilm biochemical reactors, chemostat based systems, growth-rate-controlled biofilm fermenter, the constant depth film fermenter has been discussed at the 14<sup>th</sup> International Conference on oral Biology, “Biofilms on Oral Surfaces”<sup>109</sup>

## AIM AND OBJECTIVE

Dental Plaque accumulates preferentially at stagnant sites that afford protection from the vigorous removal forces that apply in the mouth. From the oral surfaces, by mechanical means such as tooth brushing, dental flossing, results in a short term remission of the signs of gingivitis, periodontitis, and dental caries.

The development of chemotherapeutic agents capable of inhibiting dental plaque formation has been of great interest to dental researchers and clinical dentist over past decade.

Bacteria growing in dental plaque also display increased resistance including those used in dentrifices and mouth rinses. For example biofilm inhibitory concentration for Chlorhexidine and amine fluoride was 300 and 75 times greater respectively. Confocal microscopy of in situ established natural biofilms showed that Chlorhexidine only affected the outer layers of cells in 24 and 48h plaque biofilms. Biofilms of oral bacteria are also more resistant antibiotics like Amoxicillin, Doxycycline, and Metranidazole.<sup>110</sup>

The literature revealed that no detailed Pharmacognostic studies, its effect on dental plaque were not been studied. So we planned to develop easily available plant based therapeutic agent for dental plaque without mutagenic effect in the oral mucosa.

## **Aim**

To study the Pharmacognostic, preliminary phytochemical studies, and preliminary invitro antimutagenic activity and its effect on invitro dental plaque biofilm model of the ethanolic extract of the leaves of *W.tinctoria*

## **Objective**

The objective of the study was divided into four parts:

### **Part I : Pharmacognostic studies**

Identification, collection and authentication of the leaves of *W.tinctoria*.

Detailed Pharmacognostic study on leaves of *W.tinctoria* quantitative microscopy and other parameters.



## **Part II – Phytochemical Studies**

Preliminary phytochemical analysis on the crude leaf powder and on their different extracts of the leaves of ***W.tinctoria***.

Isolation of  $\beta$  amylin and identification.

HPTLC finger prints of ethanolic and ethyl acetate extracts of leaves of ***W.tinctoria***.

## **Part III (Section – A) - Antimutagenic Activity**

To evaluate antimutagenic activity of ethanolic extract of leaves of *W.tinctoria* using *Drosophila melanogaster* for visible morphological changes.

## **Part III(Section – B) - Effect on Invitro Dental Plaque Biofilm Model**

To evaluate the effect of ethanolic extract of the leaves of ***W.tinctoria*** on the artificially developed microbial plaque formed by the *S.mutans* on Nichrome wires.

# PHARMACOGNOSTIC STUDIES

## SECTION - A

### MACROSCOPICAL STUDY OF *Wrightia tinctoria* (Roxb.)R.Br.<sup>111,112</sup>

*Wrightia tinctoria* is a small deciduous tree with a light gray with scaly smooth bark belonging to the family **Apocynaceae**. Wrightia is named after a Scottish Physician and Botanist William Wright (1740-1827)

#### Plant Taxonomy (Scientific Classification)<sup>113</sup>

Kingdom	:	Plantae-plants
Subkingdom	:	Tracheobionta – vascular plants.
Phyllum/division	:	Angiospermae
Super division	:	Spermatophyta – seed plants
Class	:	Magnoliopsida (Dicotyledonous)
Sub Class	:	Rosidae
Order	:	Gentianales
Family	:	Apocynaceae
Sub family	:	Mimosoidae
Genus	:	Wrightia
Species	:	tinctoria

Synonym<sup>114</sup> : Wrightia rothii  
Nerium tinctorium Roxb.  
Dyers's Oleander

Common Names<sup>113</sup> : Tontampalai  
Veppalai  
Vetpalai  
Irumpalai

**Vernacular Names**<sup>115,114,117,93,24,94</sup>

English : Pala indigo plant  
Gujerati : Runchallaodudhlo, Dudhlo  
Hindi : Mitha inderaju, Dudhi, Indaraju  
Kannada : Kirikodasige, Bepalli, Kodamurki  
Malayalam : Kotakappala, Ayyappala, tinnampala  
Marathi : Kala Kuda  
Sanskrit : Svetakutajah, Hyamaraka  
Tamil : **Vetpalai, Thontampalai, Kodisha,[vp+pKII,**  
Telugu : Jaddapala, Tedlapala, Kala kuta, Jedda pala

Bengali : Indrajau

Oriya : Pita karumn, Dudhokriya

## **ORIGIN**

India and Burma.

## **Geographical Distribution**<sup>116,93</sup>

It is distributed throughout India upto 1,200 m. Widely distributed in Western Ghats of Madras State, Madhya Pradesh, Rajamuntry Hills, Carnatic, the Circars, and Rajasthan, and peninsular India.

## **Habit and Habitat**<sup>112, 117,116</sup>

The tree ***W.tinctoria*** is a small or middle sized tree generally up to 1.8m tall and often under 60cm, ascending to an altitude of 1200m in the hills. The tree is moderate light demander and is often found as an undergrowth species in deciduous forest. It requires a mixture of peat, loam, and sand and is propagated by seeds or cuttings which readily root in sand under the sun. It produces root-suckers. The growth is slow to moderate. The annual increase in girth being 1.16-2.3 cm. White latex is collected from the fresh leaves. The tree sheds its leaves during the cold season. About the beginning of April fresh leaves are formed together with the flowers. The seeds ripen in the following

January. It's wood is remarkably white, closely grained, and coming near to ivory than that of any other. (Plate -1, Fig -2).

## **Description**

### **Leaves**

- Colour** : Pale green
- Odour** : No characteristic odour.
- Taste** : Pungent
- Texture** : Smooth

Elliptic ovate, elliptic lanceolate, obovate oblong, or oblong to lanceolate, short petioled, 3-6 inches long and 1-2 1/2 inches broad, acuminate or cordate apex, acute or rounded base with 6-12 pairs of main nerves which are faint till the leaf matures. The leaves in this tree yields a blue dye namely Pala Indigo. Petiole 1/8 to 1/6 inches long. (Plate -2,3

Fig -2).

### **Latex**

When the leaves were picked white colored latex was oozing from the leaf and stem. (Plate – 4)

## **Flowers**

Flowers are numerous, bisexual, actinomorphic, hypogynous white color fragrance flowers, five stamens which are connivent. From a distance, the white flowers may appear like snow flakes on a tree. During dry season from the second week of April to the first week of June. It shows sparse flowering occasionally during the rainy season from the second week of July to the second week of August.(Plate –5, Fig –2)

## **Calyx**

Glabrous, glandular inside, five green colored sepals.

## **Corolla**

Gamopetalous, regular salver shaped, with a short slightly gibbous tube, crowning of the mouth of the corolla tube is a well developed corona of numerous whitish ramous linear scales. Corolla is divided into five elaborate petals.

## **Filaments**

Epipetalous, short, rigid, inserted within the mouth of the tube or within the corolla.

## **Anthers**

Exserted, fairly large, arrow shaped.

**Pollen grains**

Creamy white and sticky throughout the flower life.

**Pistil**

Bicarpellary, ovaries seemingly united. Both ovaries have a common style and stigma which are situated slightly below the level of the anthers and completely concealed by the conical filaments.

**Stigma**

Bilobed stigma with transparent gluten by which the pistil adheres to the inside of the anthers.

**Fruit**

Fruits of two, initially green and becomes brown when mature, very long, follicles in pairs, pendulous, slender, cylindrical in nature, glabrate or smooth, slightly tapering at both ends and coadhering at their tips, length 6-18 inches, diameter 1/4 to 1/3 inch.(Plate – 6, Fig – 2)

**Seeds**

Numerous, 1/2-3/4 inch long, linear, glabrous, slender, pointed at apex with a fine silky hairs often more than 1 1/2 inch long, nonendospermous, embryo inverse. (Plate – 7, Fig – 2)

## **Bark**

Pale, grey, smooth thin bark abounding in yellow milky juice with opposite divaricated branches, reddish brown in color.

## **Wood**

Uniformly white when first exposed, turning ivory colored with age, even grained, lustrous, smooth, straight or somewhat wavy or curly, even-textured. (Plate - 8)

## **Mean of propagation**

Seedlings and by vegetative propagation.

# **SECTION – B**

## **MICROSCOPICAL STUDIES<sup>118,119,120,121,122</sup>**

### ***Wrightia tinctoria* (Roxb.) R. Br.**

## ***Collection of Specimens***

The plant specimens were collected from Reserve Line Area of Madurai District of Tamil Nadu during August 2008. Care was taken to select healthy plants and for normal organs. The leaves were cut and removed from the plant and fixed in FAA (Formalin-5 ml + Acetic acid – 5 ml + 70% ethyl alcohol-90 ml). After 24 hrs of fixing, the specimens



were dehydrated with graded series of tertiary-butyl alcohol. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60<sup>0</sup>c), until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

### ***Sectioning***

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the sections was 10-12 µm. After dewaxing the sections were stained with toluidine blue. Since toluidine blue is a polychromatic stain, the staining results were remarkably good and some cyto chemical reactions were also obtained. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc., Where ever necessary, section were also stained with safranin and fast-green and potassium iodide (for starch).

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the

surface of leaf) as well as clearing of leaf with 5% sodium hydroxide and epidermal peeling by partial maceration employing jeffrey's maceration were prepared. Glycerine mounted temporary preparations were made for macerated/cleared materials.

### ***Photomicrographs***

Microscopic descriptions of tissues are supplemented with micrographs where ever necessary. Photographs of different magnifications were taken with Nikon labphot 2 microscope unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background.

## **MICROSCOPY OF THE LEAF**

### **MIDRIB**

Thick wide pot shaped. (Plate - 9, 10, Fig - 3 ). The midrib is 1.5 mm in vertical axis and 1.3 mm in horizontal axis. It has thin epidermal layer of semicircular cells.

### **ADAXIAL SIDE**

Shallow and wide slight hump, has four or five layers of collenchymas cells, apostamatic, amoeboid in outline, anticlinal walls are thick and wavy. (Plate – 11)

### **ABAXIAL SIDE**

Homogenous parenchyma cells are circular and compact with intracellular spaces, abaxial epidermis is stomatiferous, paracytic type, (60-70  $\mu\text{m}$  long, 30-40  $\mu\text{m}$  wide). Epidermal cells are amoeboid with wavy anticlinal walls. (Plate - 11)

## GROUND TISSUE

Wide circular cells which are **latricifers** or latex secreting cells which are random in distribution without any inclusions.

## VASCULAR BUNDLE

Wide, deep, and U Shaped.

**Xylem** – Closely arranged rows of xylem elements, **five** in each rows, they are angular and thick walled. **Meta xylem** is 40 µm wide.

**Phloem** - Small circular units both outer and inner side of the xylem. (wider)

## LAMINA

220 µm thick, adaxial epidermis which are thick walled, tabular, 20 µm thick. Abaxial epidermis is thick walled **squarish** cells and are 15 µm thick. (Plate -12)

## MESOPHYLL

Short narrow cylindrical **palisade** cells which are 50 µm height. **Calcium oxalate** druses **abundant** under Polarized Light Microscope (50 µm in diameter). **Spongy mesophyll** is five to six layered, spherical or loosely linked with each other forming wide air chambers. Small lateral veins in the upper portion with small clusters of xylem and few phloem and bundle sheath of parenchyma. (Plate -13)

## VENATION

Vein islets are wide and distinct varying in size and shape. Well developed, vein terminals invariantly forked repeatedly forming **dendroid configuration**. (Plate – 14).

## PETIOLE

Pot shaped, 5 mm horizontally and 4 mm vertically. The adaxial lateral twigs are not prominent. Epidermis consists of continuously arranged squarish cells. The ground tissue is totally parenchymatous lacking collenchymas. The cells are circular, fairly thick walled and compact.

The vascular system has one wide bowl shaped main strand and two small circular lateral accessory strands. (1.4 µm horizontally and 400 µm thick.) The xylem elements are thick walled and angular and are arranged in close. Phloem occurs in discrete strands both on the inner and outer sides of the Xylem. It has central cluster of xylem surrounded by phloem.

(Plate – 15)

## POWDER MICROSCOPY OF LEAF POWDER OF *W.tinctoria*

### Organoleptic Characters

Nature	:	Coarse
Color	:	Green
Odour	:	No distinct odour
Taste	:	Pungent

The powder microscopy of the leaf powder of *W.tinctoria* reveals the following characters.

Epidermal cells with stomata.

Paracytic type of stomata

Palisade cells with epidermal cells.

Fragment of leaf showing venation pattern

Calcium oxalate druses were seen in abundance in mesophyll tissue.

The druses are 25 – 40 µm thick. (Plate No – 16)

Epidermal cells with wavy anticlinal walls

Spongy parenchyma

Spiral and annular cells

**SECTION – C**

**QUANTITATIVE MICROSCOPY OF LEAVES OF**

***W.tinctoria*<sup>123,124,125,126,127</sup>**

***MICROSCOPIC SCHEDULES***

The vein islet number, vein terminal number, stomatal number and stomatal index were determined on fresh leaves using standard procedures.

***A. VEIN ISLET NUMBER AND VEIN TERMINAL NUMBER***

The term vein islet is used to denote the minute area of photosynthetic tissue encircled by the ultimate divisions of the conducting strands. The number of vein islets per sq. mm., area is called vein islet number.

Vein terminal number may be defined as the number of vein terminals present in one sq., mm. area of the photosynthetic tissue.

## ***DETERMINATION OF VEIN ISLET NUMBER AND VEIN TERMINATION NUMBER***

Leaf lets were cleared in chloral hydrate, stained and mounted on a slide.

A camera lucida is set up and by means of a stage micrometer the paper in divided into squares of  $1\text{mm}^2$  using a 16mm objective. The stage micrometer is then replaced by the cleared preparation and the veins are traced in four contiguous squares, either in a square  $2\text{mm} \times 2\text{mm}$  (or) rectangle  $1\text{mm} \times 4\text{mm}$ .

When counting, it is convenient to number each vein-islet on the tracing. Each numbered area must be completely enclosed by veins, and those which are incomplete are excluded from the count if cut by the top and left-hand sides of the square (or) rectangle but included if cut by the other two sides.

Ten readings for vein islet and vein termination number were recorded.



Table - 3

**VEIN ISLET NUMBER AND VEIN TERMINATION NUMBER OF *W.tinctoria***

Observation number	Vein Islet Number	Vein Termination Number
1	7	9
2	8	10
3	6	8
4	7	9
5	9	7
6	8	8
7	7	9
8	6	8
9	9	10
10	8	9

Range	Minimum	Average	Maximum
Vein islet Number	6	6.8	9
Vein Termination Number	7	8.7	10

**STOMATAL NUMBER**

Stomatal number is defined as the number of stoma present in one square mm area of the photosynthetic tissues.

## **METHOD**

Using fresh leaves, replicas of leaf surface may be made which are satisfactory for the determination of stomatal number and stomatal index. An approximate 50% gelatin and water gel is liquified on a water – bath and smeared on a hot slide. The fresh leaf is added, the slide inverted and cooled under a tap and after about 15-30 min the specimen is stripped off.

The imprint on the gelatin gives a clear outline of epidermal cells, stomata and trichomes.

Table - 4

STOMATAL NUMBER

Observation Number	Lower epidermis
1	78
2	80
3	81
4	82
5	79
6	84
7	83
8	75
9	80
10	77

Range	Minimum	Average	Maximum
Lower epidermis	75	79.9	84

## **B. STOMATAL INDEX**

It is the percentage, which the numbers of stomata from the total number of epidermal cells, each stoma being counted as one cell.

$$I, \text{ Stomatal index} = S/S+E \times 100$$

Where       $S$  = Number of stomata per unit area

$E$  = Number of epidermal cells in the same unit area

## **DETERMINATION OF STOMATAL INDEX**

The procedure adopted in the determinations of stomatal number was observed under high power (45 x). The epidermal cells and the stomata were counted. From these values the stomatal index was calculated using the above formula.

**Table – 5**  
**STOMATAL INDEX**

Observation Number	Lower epidermis
1	19.31
2	18.3
3	16.5
4	17.51
5	20.1
6	18.9
7	17.65
8	19.32
9	20.12
10	18.75

Range	Minimum	Average	Maximum
Lower epidermis	17.51	18.64	20.12

## **DETERMINATION OF PALISADE RATIO**

Pieces of leaves were cut off from various regions between the midrib and the margin. These pieces of leaves were cleared and mounted on a slide. Camera lucida and drawing board were arranged. Then the slide was observed under high power. Four contiguous epidermal cells devoid of trichomes and stomata were traced. The total number of palisade cells were counted and divided by four. Ten such readings taken and the average was calculated.

**Table - 6**

**PALISADE RATIO**

<b>Observation Number</b>	<b>Palisade Ratio</b>
1	8
2	7
3	6
4	7
5	5
6	9
7	6
8	7
9	7
10	8

<b>Range</b>	<b>Minimum</b>	<b>Average</b>	<b>Maximum</b>
Palisade Ratio	5	7	9

## QUANTITATIVE SCHEDULES FOR LEAVES OF

### *W.tincoria*

#### **ASH VALUE**

The ash values were determined by using air dried powder of the leaf as per the official method.

#### **TOTAL ASH**

Two grams of the air dried leaf powder was accurately weighed in a platinum crucible separately. The powder was scattered into a fine even layer on the bottom of the crucible and incinerated by gradually increasing the temperature not exceeding 450°C, until free from carbon. Then it was cooled and weighed for constant weight. The percentage of ash with reference to the air dried powder was calculated.

#### **WATER SOLUBLE ASH**

The ash obtained from the total ash procedure was boiled with 25 ml of water for 5 minutes and the insoluble matter was collected on an ash less filter paper and washed with hot water. Then it was ignited for 15 minute at a temperature not exceeding 450°C. The weight of the insoluble matter was subtracted from the weight of the total ash. The difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried powder.



### **ACID INSOLUBLE ASH**

The ash obtained from the total ash was boiled for five minutes with 25 ml of dilute hydrochloric acid. The insoluble matter was collected in a tarred sintered glass crucible. The residue was washed with hot water, dried and weighed. The percentage of acid insoluble ash with reference to the air dried drug was calculated.

**Table - 7**

#### **ASH VALUES FOR THE LEAVES OF *W.tinctoria***

Observation Number	Total Ash (%)	Acid Insoluble ash (%)	Water soluble Ash (%)
1	8.63	0.98	-
2	9.24	0.85	-
3	8.41	0.67	-
4	10.55	0.93	-
5	8.84	0.75	-
6	10.21	-	6.19
7	9.75	-	5.93
8	8.78	-	6.12
9	9.76	-	5.59
10	9.86	-	5.98

Range	Total ash	Acid Insoluble ash (%)	Water soluble Ash (%)
Minimum	8.41	0.67	5.59
Average	9.40	0.83	5.96
Maximum	10.21	0.98	6.12

### ***Determination of Loss on Drying***

For the determination of loss on drying, the method described by wallis was followed.

One gram of the powdered leaf was accurately weighed in a tarred Petri dish, previously dried under the conditions specified in IP '96. The powder was distributed as evenly as practicable, by gentle sidewise shaking. The dish was dried in an oven at 100 – 105<sup>0</sup> c for 1 hour. It was cooled in a desiccator and again weighed. The loss on drying was calculated with reference to the amount of the dried powder taken.

**Table - 8**

**PERCENTAGE LOSS ON DRYING FOR THE LEAVES OF *W.tinctoria***

Observation Number	Loss on Drying (%) W/W
	Leaf
1	8.3
2	7.5
3	7.6
4	8.12
5	7.48

Material	Minimum	Average	Maximum
Leaf	7.48	7.8	8.3

**EXTRACTIVE VALUES**

***Petroleum Ether Soluble Extractive Value***

Five gram of the coarsely powdered leaf was macerated separately with 100 ml of petroleum ether in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of petroleum ether. 25 ml, of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish,

dried at 105°C and weighed. The percentage of the petroleum ether soluble extractive value was calculated with reference to the air dried powder.

#### ***Benzene Soluble Extractive Value***

Five gram of the coarsely powdered leaf was macerated separately with 100 ml of benzene in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of benzene. 25 ml, of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the benzene soluble extractive value was calculated with reference to the air dried powder.

#### ***Ethyl Acetate Soluble Extractive Value***

Five gram of the coarsely powdered leaf was macerated separately with 100 ml of ethyl acetate in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of ethyl acetate. 25 ml, of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the ethyl acetate soluble extractive value was calculated with reference to the air dried powder.

#### ***Chloroform Soluble Extractive Value***

Five gram of the coarsely powdered leaf was macerated separately with 100 ml of chloroform in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of chloroform. 25 ml, of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried

at 105°C and weighed. The percentage of the chloroform soluble extractive value was calculated with reference to the air dried powder.

#### ***Ethanol Soluble Extractive Value***

Five gram of the coarsely powdered leaf was macerated separately with 100 ml of ethanol in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of ethanol. 25 ml, of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the ethanol soluble extractive value was calculated with reference to the air dried powder.

#### ***Water Soluble Extractive Value***

Five gram of the coarsely powdered leaf was macerated separately with 100 ml of chloroform water in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of chloroform water. 25 ml, of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the chloroform water soluble extractive value was calculated with reference to the air dried powder.

**Table - 9**

**EXTRACTIVE VALUES (INDIVIDUAL SOLVENTS)**

<b>Solvents</b>	<b>Extractive Value (%)</b>
	<b>Leaf</b>
Petroleum ether	1.3
Benzene	0.06
Ethyl acetate	0.82
Chloroform	1.89
Ethanol	2.12
Water	5.4

***Extractive Values***

***By using solvents successively with increasing order of polarity***

Five grams of the coarsely powdered leaf was extracted continuously in soxhlet apparatus for six hours individually, separately with solvents of increasing order of polarity. After six hours the solvents was removed and evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the individual solvent soluble extractive value was calculated with reference to the air dried powder.

**Table – 10**

**EXTRACTIVE VALUES (SUCCESSIVE SOLVENTS)**

<b>Solvents</b>	<b>Extractive Value (%)</b>
	<b>Leaf</b>
Petroleum ether	1.42
Benzene	0.32
Ethyl acetate	0.62
Chloroform	1.22
Ethanol	2
Water (reflux)	4.01

# PHYTOCHEMICAL STUDIES

## SECTION – A

The plant ***W.tinctoria*** was collected at Reserve Line area of Madurai District in Tamil Nadu during the first week of August and it was Authenticated by the Taxonomist. The leaves portion was washed thoroughly and dried in shadow. The shadow dried leaves were powdered separately and then subjected to the following preliminary phytochemical studies.

### ORGANOLEPTIC EVALUATION

Nature of the Powder	:	Coarse
Colour	:	Pale green
Odour	:	No characteristic odour
Taste	:	Slightly bitter
Shaken with Water	:	Frothing absence
Pressed in between two filter paper	:	No oily mark on the paper.



## PHYTOCHEMICAL STUDIES FOR THE LEAF POWDER OF *W.tinctoria*<sup>128,129,130</sup>

Powdered Materials and their individual extracts obtained from different increasing polarity were subjected to the following chemical test and the results were presented in the table.

### ***Test for Alkaloids***

#### ***Various procedures to liberate alkaloids***

Powdered drug was mixed thoroughly with 1 ml of 10% ammonia solution and then extracted for 10 minutes with 5 ml methanol, under reflux. The filtrate was then concentrated.

Powdered drug was mixed thoroughly with 1 ml of 10% sodium carbonate solution and then extracted for 10 minutes with 5 ml methanol, under reflux. The filtrate was then concentrated.

Powdered drug was ground in a mortar for about 1 minute with 2 ml of 10% ammonia solution and then thoroughly mixed with 7 gram basic aluminum oxide. The mixture was then loosely packed in to a glass column and 10 ml chloroform was added, eluted, dried and methanol was added.

Powdered drug was shaken for 15 minute with 15 ml of 0.1 N sulphuric acid and then filtered. The filter was washed with 0.1 N sulphuric acid to a volume of 20 ml filtrate; 1 ml concentrated ammonia was then added. The mixture was then shaken with two portions of 10 ml diethyl ether. The ether was dried

over anhydrous sodium sulphate, filtered and evaporated to dryness and the resulting residue was dissolved in methanol.

Powdered drug was mixed with one gram of calcium hydroxide and 5 ml of water, made into a smooth paste and set aside for 5 minutes. It was then evaporated to dryness in a porcelain dish on a water bath. 20 ml of 90% alcohol was added, mixed well and then refluxed for half an hour on a water bath. It was then filtered and the alcohol was evaporated. To that dilute sulphuric acid was added.

The above made extracts were tested with various alkaloid reagents and the results were as follows.

- |                                 |   |                           |
|---------------------------------|---|---------------------------|
| <b>1. Mayer's reagent</b>       | - | Cream color precipitate   |
| <b>2. Dragendorff's reagent</b> | - | Reddish brown precipitate |
| <b>3. Hager's reagent</b>       | - | Yellow precipitate        |
| <b>4. Wagner's reagent</b>      | - | Reddish brown precipitate |

#### ***Test for purine group (Murexide test)***

The residue obtained after the evaporation of chloroform was treated with 1 ml of hydrochloric acid in a porcelain dish and 0.1g of potassium chlorate was added and evaporated to dryness on water bath. Then the residue was exposed to the vapours of dilute ammonia solution. No purple colour was obtained indicating the **absence** of purine group of alkaloids.

#### ***Test for Carbohydrates***

##### ***Molisch's test***

The aqueous extract of the powdered material was treated with alcoholic solution of  $\alpha$  - naphthol in the presence of sulphuric acid.

Purple colour was obtained indicating the **presence** of carbohydrates.

### ***Fehling's test***

The aqueous extract of the powdered material was treated with Fehlings I and II solution and heated on boiling water bath.

Reddish brown precipitate was obtained indicating the **presence** of free reducing sugars.

### ***Benedict's test***

The aqueous extract of the powdered drug was treated with Benedict's reagent and heated over a water bath.

Reddish brown precipitate was obtained indicating the **presence** of reducing sugars.

### ***Test for Glycosides***

#### ***General test***

##### ***Test A***

200 mg of the powdered drug was extracted with 5 ml of dilute sulphuric acid by warming on a water bath, filtered and neutralised with 5% sodium hydroxide solution. Then 0.1 ml of Fehlings solution A and B were added, until it becomes alkaline and heated on a water bath for 2 mts.

##### ***Test B***

200 mg of the powdered drug was extracted with 5 ml of water instead of sulphuric acid. Boiled and equal amount of water was added instead of sodium hydroxide solution. Then 0.1 ml of Fehlings solution A and B were added, until it becomes alkaline and heated on a water bath for 2 mts.

The quantity of red precipitate formed in test A is greater than in test B indicating the **presence** of glycosides.

### ***Anthraquinones***

#### ***Borntrager's test***

The powdered leaf was boiled with dilute sulphuric acid, filtered and to the filtrate benzene was added and shaken well. The inorganic layer was separated and ammonia solution was added slowly.

No color reaction was observed in ammoniacal layer indicating the **absence** of anthracene derived glycosides.

#### ***Modified Borntrager's test***

About 0.1 gram of the powdered leaf was boiled for two minutes with dilute hydrochloric acid and few drops of ferric chloride solution was added, filtered while hot and cooled. The filtrate was then extracted with benzene and the benzene layer was separated. Equal volume of dilute ammonia solution was added to the benzene extract and shaken well.

No color was observed in ammoniacal layer indicating the **absence** of anthracene derived glycosides.

#### ***Test for cyanogenetic glycosides***

Small quantity of the powdered leaf was placed in a stoppered conical flask with just sufficient water to cover it. A sodium picrate paper strip was

inserted through the stopper so that it was suspended in the flask and it was set aside for 2 hours in a warm place.

No change in the colour of the sodium picrate paper was observed indicating the **absence** of cyanogenetic glycosides

### ***Test for cardiac glycosides***

#### ***Keller Killiani test***

About 1 gram of the powdered leaf was boiled with 10 ml of 70% alcohol for two minutes, cooled and filtered. To the filtrate 10 ml of water and 5 drops of solution of lead sub acetate were added and filtered. The filtrate was then extracted with chloroform and the chloroform layer was separated and evaporated to dryness. The residue was dissolved in 3 ml of glacial acetic acid containing a trace of ferric chloride. To this 3 ml of concentrated sulphuric acid was added to the sides of the test tube carefully.

No reddish brown layer acquiring bluish green color after standing was observed indicating the **absence** of deoxy sugars of cardiac glycosides.

#### ***Raymond Test***

To the alcoholic extract of the leaf, hot methanolic alkali was added.

No Violet color was produced indicating the **absence** of cardiac glycosides.

#### ***Legal's Test***

To the alcoholic extract of the powdered drug, pyridine and alkaline sodium nitro pruside solution were added.

No blood red color was formed indicating the **absence** of cardiac glycosides.

### ***Coumarin glycosides***

A small amount of powdered leaf was placed in test tube and covered with a filter paper moistened with dilute sodium hydroxide solution. The covered test tube was placed on water bath for several minutes. Then the paper was removed and exposed to UV light.

No green fluorescence was observed indicating the **absence** of coumarin glycosides.

### ***Test for Phytosterols***

The powdered leaf was first extracted with petroleum ether and evaporated. The residue obtained was dissolved in chloroform and tested for sterols.

### ***Salkowski Test***

Few drops of concentrated sulphuric acid were added to the above solution, shaken well and set aside.

The chloroform layer of the solution turned red in color indicating the **presence** of sterols.

### ***Libermann – Burchard's Test***

To the chloroform solution few drops of acetic anhydride was added and mixed well 1 ml of concentrated sulphuric acid was added through the sides of the test tube and set aside for a while.

A brown ring was formed at the junction of the two layers and the upper layer turned green indicating the **presence** of sterols.

### ***Test for Saponins***

About 0.5 gram of the powdered leaf was boiled gently for 2 minute with 20 ml of water and filtered while hot and allowed to cool. 5 ml of the filtrate was then diluted with water and shaken vigorously.

Absence of frothing occurred indicating the **absence** of saponins.

### ***Test for Tannins***

To the aqueous extract of the powdered leaf, few drops of ferric chloride solution were added.

Bluish black color was produced, indicating the **presence** of tannins.

### ***Gold beater's skin test***

2% hydrochloric acid was added to a small piece of gold beater skin and rinsed with distilled water and placed in the solution to be tested for five minutes. Then washed with distilled water and transferred to a 1% ferrous sulphate solution.

Formation of brown color indicates the **presence** of tannins.

### ***Test for Proteins and Free Aminoacids***

#### ***Millon's test***

The aciduous alcoholic extract of the powdered leaf was heated with Millon's reagent.

The colour was changed to red on heating indicating the **presence** of proteins.

#### ***Biuret test***

To the alcoholic extract of the powdered leaf 1 ml of dilute sodium hydroxide was added. Followed by this one drop of very dilute copper sulphate solution was added.

Violet color was obtained indicating the **presence** of proteins.

#### ***Ninhydrin Test***

To the extract of the powdered drug, ninhydrin solution was added, and boiled.

Formation of violet color indicating the **presence** of Aminoacids

### ***Test for Mucilage***

To the aqueous extract of the powdered leaf, ruthenium red solution was added.

No Reddish pink color was produced indicating the **absence** of Mucilages.

### ***Test for Flavonoids***



### ***Shinoda Test***

A little amount of the powdered leaf was heated with alcohol and filtered. To the alcoholic solution a few magnesium turnings and few drops of concentrated hydrochloric acid were added, and boiled for 5 minutes.

Purple color was obtained indicating the **presence** of flavanoids.

### ***Alkaline reagent test***

To the alcoholic extract of the powdered leaf, few drop of sodium hydroxide solution was added.

Yellow color was formed, indicating the **presence** of flavanoids

### ***Zinc Hydrochloride Test***

To the alcoholic extract, mixture of zinc dust and concentrated hydrochloric acid was added.

Red color formed indicating the **presence** of flavanoids.

### ***Test for Terpenoids***

The powdered leaf was shaken with petroleum ether and filtered. The filtrate was evaporated and the residue was dissolved in small amount of chloroform. To the chloroform solution tin and thionyl chloride were added.

Pink color was obtained indicating the **presence** of Terpenoids.

[

### Test for Volatile Oil

About 100 gram of fresh leaves, were taken in a volatile oil estimation apparatus (Cocking Middleton apparatus) and subjected to hydro distillation for four hours.

No Volatile oil was obtained indicating the **absence** of volatile oil.

### Test for Fixed Oil

A small amount of the powdered leaf was pressed in between in the filter paper and the paper was heated in an oven at 105°C for 10 minutes.

No translucent greasy spot occurred indicating the **absence** of fixed oil.

**Table - 11**

#### **RESPONSE TO THE PRELIMINARY PHYTOCHEMICAL SCREENING FOR THE CRUDE LEAF POWDER OF *W.tinctoria***

S.NO	TEST	OBSERVATION
<b>I.</b>	<b>ALKALOIDS</b>	
	Mayer's reagent	+
	Dragendorff's reagent	+
	Hager's reagent	+
	Wagner's reagent	+
<b>II</b>	<b>CARBOHYDRATES</b>	
	Molisch's test	+
	Fehling's test	+
	Benedict's test	+
<b>III</b>	<b>GLYCOSIDES</b>	
	General Test	+
	Anthraquinone	-
	Cardiac	-

	Cyanogenetic	-
	Coumarin	-
<b>IV</b>	<b>PHYTOSTEROLS</b>	
	Salkowski test	+
	Lieberman Burchard's test	+
<b>V</b>	<b>SAPONINS</b>	-
<b>VI</b>	<b>TANNINS</b>	+
<b>VII</b>	<b>PROTEINS AND FREE AMINO ACIDS</b>	
	Millon's test	+
	Biurett test	+
<b>VIII</b>	<b>GUMS AND MUCILAGE</b>	-
<b>IX</b>	<b>FLAVONOIDS</b>	
	Shinoda test	+
<b>X</b>	<b>TERPENOIDS</b>	+
<b>XI</b>	<b>VOLATILE OIL</b>	-
<b>XII</b>	<b>FIXED OIL</b>	-

The above described tests were also performed on the different extracts of leaf powder of *W.tinctoria* and the results were as follows,

**Table – 12**  
**RESPONSE TO THE PRELIMINARY PHYTOCHEMICAL SCREENING FOR**  
**THE DIFFERENT EXTRACTS OF LEAF OF *W.tinctoria***

Tests	Petroleum ether extract	Benzene extract	Ethyl acetate extract	Chloroform extract	Ethanollic extract	Aqueous extract
<b>ALKALOIDS</b>						
Mayers Reagent	-	-	-	-	+	-
Dragendorffs reagent	-	-	-	-	+	-
Hagers reagent	-	-	-	-	+	-
Wagners reagent	-	-	-	-	+	-
<b>CARBOHYDRATES</b>						
Molishch's Test	-	-	-	-	+	+
Fehlings Test	-	-	-	-	+	+
Benedicts Test	-	-	-	-	+	+
<b>GLYCOSIDES</b>						
General Test	-	-	-	-	+	+
Anthraquinone	-	-	-	-	-	-
Cardiac	-	-	-	-	-	-
Cyanogenetic	-	-	-	-	-	-
Coumarin	-	-	-	-	-	-
<b>PHYTOSTEROLS</b>						-
Salkowski Test	+	+	+	+	-	-

Libermann Burchard Test	+	+	+	+	-	-
<b>SAPONINS</b>	-	-	-	-	-	-
<b>TANNINS</b>	-	-	-	-	+	+
<b>PROTEINS &amp; FREE AMINO ACID</b>						
Millons test	-	-	-	-	-	+
Biuret test	-	-	-	-	-	+
Ninhydrin test	-	-	-	-	-	+
<b>GUMS &amp; MUCILAGE</b>	-	-	-	-	-	-
<b>FLAVONOIDS</b>						
Shinoda test	-	-	-	-	+	-
Alkaline Reagent test	-	-	-	-	+	-
Zinc hydrochloric acid test	-	-	-	-	+	-
<b>TERPENOIDS</b>	+	-	+	+	-	-
<b>FIXED OIL</b>	-	-	-	-	-	-

“+” Indicate Positive reaction      “-” Indicate Negative reaction

## SECTION – B

### THIN LAYER CHROMATOGRAPHY OF ETHANOLIC AND ETHYL

#### ACETATE EXTRACT OF *W.tinctoria*<sup>131,132,133</sup>

Among the various methods of separating and isolating plant constituents the “chromatographic Procedure” originated by Tswett is one of the most useful techniques of general application. All finely divided solids have the power to adsorb other substances are capable of being absorbed some much more readily than others. Thin phenomenon of selective adsorption is the fundamental principle of chromatography.

#### Principle

When a mixture of compound is spotted on a TLC plate the compound which readily soluble and not strongly absorbed moves up readily along with

this solvent. Those which are not so soluble, and are more strongly absorbed moves up less readily to the separation of the compound.

The advancement of the TLC techniques has provided the organic chemists and biochemists a tool which combines in itself sensitivity and rapidity compound to the conventional paper chromatographic technique.

### **Application of substance mixture for separation**

The substance mixture was taken in a capillary tube and it was spotted on TLC plated 1cm above its bottom end the start points were equally sized as for as possible.

### **Development of chromatogram**

The plates were developed in a chromatographic tank by using a range of solvents from non-polar to polar as a mobile phase. The plates were allowed to develop  $\frac{3}{4}$  of the length and then removed. The solvent front was immediately marked and the plates were allowed to dry. Then the plates were examined visually or under UV (or) sprayed with different reagents.

The  $R_f$  value was calculated by the following formula.

$$R_f = \frac{\text{Distance travelled by solute front}}{\text{Distance traveled by solvent front}}$$

Stationary Phase : Silica Gel G

Mobile Phase :Toluene:Acetonitrile (3:1)

Detecting agent : Iodine chamber

The  $R_f$  values and color of the developed spots were tabulated in Table - 13

**Table –13**

**TLC OF ETHANOLIC AND ETHYL ACETATE EXTRACTS OF LEAVES OF  
*W.tinctoria***

Extract	No of Spots	$R_f$ value	Color developed
Ethanolic	3	0.3 0.55 0.8	Pink Pale green Dark green
Ethyl acetate	2	0.2 0.7	Pink Yellow

**HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY**  
**ANALYSIS OF ETHANOLIC AND ETHYL ACETATE EXTRACTS OF**  
**LEAVES OF *W.tinctoria*<sup>134</sup>**

The ethanolic extract of *W.tinctoria* leaves were applied in a concentration of 10µl using CAMAG Linomat IV sample applicator on Aluminium sheets precoated with silica gel 60 F<sub>254</sub> HPTLC plates of 0.2mm thick, 5x20cm, used as a stationary phase. The plates were developed in the mobile phase, Toluene:Acetonitrile (7:3) for the ethanolic and ethyl acetate extract to a distance of 120 mm in CAMAG – Twin trough glass chamber. Then the track was scanned using CAMAG densitometer scanner II equipped with CAMAG software © 1998 CATS 3.20”, at a wavelength of 254 nm using deuterium lamp and the finger print profiles were recorded and presented in Table.

## HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

Instrument	:	CAMAG HPTLC system (Switzerland) equipped with.
Applicator	:	CAMAG Linomat IV sample applicator
Scanner	:	CAMAG densitometer scanner II: 951016
Software	:	CAMAG – SOFTWARE (C) 1988 CATS 3.20
Developing chamber	:	CAMAG – Twin trough glass chamber
HPTLC Plate	:	Silicagel 60F 254, Merck, pre coated HPTLC plate of 5 x 20cm, 0.2 mm thick.
Solvent systems	:	1) Toluene : Acetonitrile 7 : 3
Wavelength	:	254 nm
Sample	:	Ethanollic and Ethyl acetate extract of <i>W.tinctoria leaves</i>

## PROCEDURE

The spots of samples (10µl) were applied about 6mm from the edge of the TLC plates. The plates were developed upto 120 mm in the mobile phase.

## HPTLC CHROMATOGRAM

Ethanollic extract	-	6 peaks
Ethyl Acetate	-	7 peaks



TABLE – 14

HPTLC PROFILE OF ETHANOLIC EXTRACT OF *W.tinctoria leaves*

Peak Numbers	R <sub>f</sub>	Peak Height	% of total area covered by individual peak
1.	0.01	74.92	72.24
2.	0.32	4.37	4.24
3.	0.53	8.62	7.90
4.	0.63	2.62	2.13
5.	0.71	2.80	2.07
6.	0.77	6.68	10.92

**TABLE – 15**

**HPTLC PROFILE OF ETHYL ACETATE EXTRACT OF *W.tinctoria leaves***

Peak Numbers	R <sub>f</sub>	Peak Height	% of total area covered by individual peak
1.	0.02	58.16	54.47
2.	0.20	6.28	6.73
3.	0.34	8.54	8.31
4.	0.51	10.55	10.12
5.	0.62	6.09	5.31
6.	0.72	2.98	2.98
7.	0.77	7.40	12.09

## SECTION - C

### ISOLATION OF $\beta$ AMYRIN FROM THE PETROLEUM ETHER EXTRACT OF LEAVES OF *W.tinctoria*

The powdered leaves were extracted with petroleum ether.

The petroleum ether extract was concentrated and the residue was saponified using 0.1M potassium hydroxide in ethanol.

The unsaponifiable matter was benzoylated with pyridine and benzoyl chloride.

The product on repeated crystallizations from chloroform gave colorless shining plates (yield 0.09%).

The melting point of the plates were 229°C and mixed melting point 229°C.

TLC of the isolated compound was carried with the standard sample. Same R<sub>f</sub> value as that of standard further gave the suggestion that the isolated compound is  $\beta$ amyrin.<sup>52</sup>

#### TLC OF THE ISOLATED $\beta$ AMYRIN

Stationary Phase:	Precoated aluminium Plate (Silica gel 60F <sub>254</sub> )
Mobile Phase:	Ethyl acetate:Hexane (2:8)
Detecting agent:	Vanillin in Sulphuric acid
R <sub>f</sub> value:	Blue color spot (0.2)

R<sub>f</sub> of the isolated compound coincides with the standard  $\beta$  amyrin.  
Hence the spot is due to isolated  $\beta$  amyrin.

**SECTION - A**  
**ANTIMUTAGENIC EFFECT OF ETHANOLIC EXTRACT OF LEAVES**  
**OF *W.tinctoria* ON *DROSOPHILA MELANOGASTER***

**INTRODUCTION**

*W.tinctoria* is widely used in traditional medicine due to its lack of information about its mutagenic effect. So it is important to evaluate the effects of this complex mixture on mutagenic alterations. Over the last few years, mutagenic assays have been developed that are able to detect several genetic endpoints. In genetic toxicology, it is important to know whether chemical should regarded as hazardous or whether they can be considered as sufficiently safe if their mutagenic effects are nil or at least significantly below a defined minimal effect level.

The Eye and Wing spot assays have received increasing interest in the recent past. Because they are sensitive in vivo assays which are simple to carry out. They are also much less laborious, cheaper, and at the same time more informative.

In addition it is well established that *Drosophila* possess versatile system and has proved to be an assay for mutagenic screening which is easy to perform and inexpensive for the detection of promutagens.

If a mutagenic alteration takes place in one of the cells, the descendent cells will form a clone of mutant cells that can be detected as a spot in the adult tissue. The test can detect the mutagenic

chemical compound that produced a loss of heterozygosis by several chromosome breakage mechanisms such as mitotic recombination, deletion, point mutation, chromosomal loss, and aberration.<sup>139,140</sup>

## ***DROSOPHILA MELANOGASTER***

***Drosophila*** is a genus of small flies, belonging to the family Drosophilidae, whose members are often called "fruit flies" or more appropriately (though less frequently) pomace flies, vinegar flies, or wine flies, a reference to the characteristic of many species to linger around overripe or rotting fruit. These feed primarily on unripe or ripe fruit, with many species being regarded as destructive agricultural pests, especially the Mediterranean fruit fly. One species of *Drosophila* in particular, *D. melanogaster*, has been heavily used in research in genetics and is a common model organism in developmental biology. Indeed, the terms "fruit fly" and "*Drosophila*" are often used synonymously with *D. melanogaster* in modern biological literature.

### **PHYSICAL APPEARANCE OF *D.melanogaster***

Fruit flies have brick red eyes, are yellow-brown in color, and have transverse black rings across their abdomen. They exhibit sexual dimorphism; Females are about 2.5 millimeters (0.1 inches) long; males are slightly smaller and the back of their bodies is darker. Males are easily distinguished from females based on color

differences, with a distinct black patch at the abdomen, less noticeable in recently emerged flies, and the sexcombs (a row of dark bristles on the tarsus of the first leg). Furthermore, males have a cluster of spiky hairs (claspers) surrounding the reproducing parts used to attach to the female during mating.

### **LIFE CYCLE AND REPRODUCTION OF *D.melanogaster***

The developmental period for *Drosophila melanogaster* varies with temperature, as with many ectothermic species. The shortest development time (egg to adult), 7 days, is achieved at 28°C. Development times increase at higher temperatures 30 °C, 11 days due to heat stress. Under ideal conditions, the development time at 25 °C (77 °F) is 8.5 days. Under crowded conditions, development time increases, while the emerging flies are smaller. Females lay some 400 eggs (embryos), about five at a time, into rotting fruit. The eggs, which are about 0.5 millimetres long, hatch after 12–15 h (at 25 °C (77 °F)). The resulting larvae grow for about 4 days (at 25 °C) while molting twice (into 2nd- and 3rd-instar larvae), at about 24 and 48 h after hatching. During this time, they feed on the microorganisms that decompose the fruit, as well as on the sugar of the fruit itself. Then the larvae encapsulate in the [puparium](#) and undergo a four-day-long metamorphosis (at 25 °C), after which the adults eclose (emerge).<sup>135,136</sup> (Fig No 7)

### **MODEL ORGANISM IN GENETICS**

*Drosophila melanogaster* is one of the most studied organisms in biological research, particularly in genetics and developmental biology. There are several reasons:

The care and culture requires little equipment and use little space even when using large cultures, and the overall cost is low.

It is small and easy to grow in the laboratory and their morphology is easy to identify once they are anesthetized (usually with ether, carbon dioxide).

It has a short generation time (about 10 days at room temperature) so several generations can be studied within a few weeks.

It has a high fecundity (females can lay more than 800 eggs in a lifetime, i.e. one egg every 30 minutes with sufficient food).

Males and females are readily distinguished and virgin females are easily isolated, facilitating genetic crossing.

It has only four pairs of [chromosomes](#): three autosomes, and one sex chromosome.

Genetic transformation techniques have been available since 1987.

Its complete genome was sequenced and first published in 2000.<sup>138</sup>

## **SIMILARITY TO HUMANS**

About 75% of known human disease genes have a recognizable match in the genetic code of fruit flies and 50% of fly protein sequences have mammalian

analogues. An online database called Homophila is available to search for human disease gene homologues in flies and vice versa. *Drosophila* is being used as a genetic model for several human diseases including the neurodegenerative disorders Parkinson's, Huntington's, spinocerebellar ataxia and Alzheimer's disease. The fly is also being used to study mechanisms underlying aging and oxidative stress, immunity, diabetes, and cancer, as well as drug abuse.<sup>137</sup>

## **MATERIALS AND METHOD**

Stereomicroscope

Micropipettes

Diethyl ether

Stage micrometer

Eyepiece micrometer

Formaldehyde (0.5% v/v)

Ethanollic extract of leaves of *W.tinctoria* in the

concentration of 5, 10, 25, 50 µg/ml

Plastic containers.

70% v/v ethanol

Banana medium

Petridishes.

## **EXPERIMENTATION**



IIInd instar larvae from F1 generation of Normal flies were collected. (Plate No 19).

Larvae were washed with solution of 20% w/v sucrose and seeded in glass petridishes (20 larvae/vial) containing 2 gm of banana mass with 1 ml of test solution of various concentration (5, 10, 25, 50  $\mu\text{g/ml}$ ) along with negative control of solvent alone (distilled water) and standard chemical mutagen (0.5% v/v formalin).

Larvae were fed on the above medium for six hours and transferred to fresh medium for the rest of their development.

After eclosion, adult flies were collected and stored in 70% v/v ethanol for the evaluation of the mutagenic effect.

Morphological changes including eye color, spots in the wings, wing hairs, changes in the length and width of the wing, wing

shape, abdomen length, and total body length were observed under stereomicroscope.

All the experiments were carried out at room temperature. ( $\pm 12$ h day and night).

The observations were tabulated and photograph of the compared flies were presented. (Plate 20 to 24, Table – 16)

**SECTION – B**  
**INVITRO DENTAL PLAQUE BIOFILM INHIBITORY ACTIVITY OF**  
**ETHANOLIC EXTRACT OF LEAVES OF *W.tinctoria***

**INTRODUCTION**

Caries and periodontal diseases are two of the most common chronic diseases affecting mankind. These are associated with accumulation of bacterial plaque formed as a biofilm on tooth surfaces. Dental plaque is a complex multi-species biofilm community and is a direct precursor of caries and periodontal diseases. Dental caries can lead to tooth dysfunction and loss.

**STRUCTURE OF DENTAL PLAQUE**

Confocal laser scanning microscopy has confirmed that supragingival plaque has a more open architecture. Live and dead stains have suggested that bacterial vitality may vary throughout the biofilm, with the most viable bacteria present in the central part of the plaque, and lining the voids and channels. Dental plaque accumulates preferentially at stagnant sites that afford protection from the vigorous removal forces that apply in the mouth.<sup>111</sup>

## **ABOUT *STREPTOCOCCUS MUTANS***

The original version of the chemoparasitic caries theory of Miller received its 'coup de grace' by Ron Gibbons in the early 1960s, when it was shown that bacteria associated with dental caries must colonize supragingival plaque in high numbers. This shifted mainstream research to plaque *Streptococci* and in particular to mutans *Streptococci*. Animal experiments and both human cross-sectional and longitudinal studies left no doubt that mutans *Streptococci* were strongly associated with enamel caries. Dental caries was conceived as a mono-infection by mutans *streptococci*. It is a gram-positive, facultatively anaerobic bacteria commonly found in the human oral cavity and is a significant contributor to tooth decay.<sup>141</sup>

### Scientific classification

Kingdom: [Bacteria](#)  
Phylum: [Firmicutes](#)  
Class: [Cocci](#)  
Order: [Lactobacillales](#)  
Family: [Streptococcaceae](#)  
Genus: [Streptococcus](#)  
Species: ***Mutans***

Although the oral flora is quite diverse and complex, two species of mutans *Streptococci* in the biofilm, *Streptococcus mutans* and *Streptococcus sobrinus* have generally been regarded as the primary etiologic agents of dental caries in humans. These species adhere and accumulate on the tooth surface by generating extracellular polysaccharides from sucrose in the oral cavity. These particular abilities of the bacterial species are essential for the formation and structural integrity of the biofilm.<sup>11</sup>

Along with *S.mutans*, *S.sobrinus*, plays a major role in tooth decay, [metabolizing sucrose](#) to [lactic acid](#). The [acidic](#) environment created in the mouth by this process causes the highly [mineralized tooth enamel](#) to be vulnerable to decay. *S. mutans* is one of a few specialized organisms equipped with receptors that help for better adhesion to the surface of teeth. Sucrose is utilized by *S. mutans* to produce a sticky, extracellular, [dextran](#)-based [polysaccharide](#) that allows them to [cohere](#) to each other forming plaque.<sup>142</sup>

Cariogenic bacteria such as *mutans Streptococci* which produce extracellular enzymes such as glucosyltransferases and fructosyltransferases. Hence the use of antimicrobial agents in the treatment of above disease is needed.<sup>143</sup>

## BIOFILM FORMATION

Biofilms provide protection to the microbial community by regulating the environment from outside disturbances. Saliva contains a multitude of proteins that contribute to oral microbial ecology and biofilm formation. Adsorption of specific salivary proteins such as acidic proline rich proteins promotes the adherence of *S.mutans* on the enamel surfaces which in turn facilitates the dental biofilm development.<sup>144</sup>

Plaque formation occurs in two stages. The first stage is the reversible initial attachment of the bacterial cell to the pellicle coated enamel surface. The second stage involves the accumulation of *S.mutans* and is dependent upon the production of extracellular glucans produced by bacterial glucosyl-transferase enzymes from sucrose.<sup>143</sup>

## ANTIMICROBIAL AGENTS

The best way to control these negative impacts is to remove biofilm associated diseases prior to the development of dental caries. Several studies on the effect of intra orally applied antimicrobial agents such as CHX gluconate, Cetylpyridinium chloride, Parabens and Phenolic agents were shown to be effective for inhibiting biofilm development.<sup>108</sup>

## NEED FOR PLANT BASED ANTIPLAQUE AGENTS

Chlorhexidine is generally accepted as the gold standard for anti-biofilm agents in the dental field, however the use of Chlorhexidine as an anti-caries agent also has common side effects including the formation of extrinsic stain on the tooth and tongue.

Excessive use of the antimicrobial agents can result in rearrangement of the oral and intestinal flora and causes undesirable side effects such as microorganism susceptibility, vomiting, diarrhea and tooth staining. These problems implied the importance of further research for natural antiplaque agents with safety for humans and specificity for oral pathogens.<sup>145</sup>

Recently, several plants have been studied for their potential in the prevention of dental caries. **Fruit juice of Pomegranate**, aqueous extract of **neem**, and **Cranberry**, With the scientific name *Vaccinium macrocarpon*, **Sage**: Scientifically known as *Salvia officinalis*, arak (**Salvadora persica**). Essential oil of **Rosemarinus officinalis**, **Macelignan** isolated from **Myristica**

**frangans, Polygonum cuspidatum, Extract of Juglandaceae**  
are scientifically proven to have antiplaque activity.

Aqueous extracts 1% w/v of Nigerian chewing sticks derived from the African plants also possess biofilm reduction property.<sup>13</sup>

Tannin, polyphenolic compounds, which is widely distributed in the plant world has been shown to possess anticariogenic potential, and it reduces the caries formation in experimental animals as well as inhibit glucosyltransferase activity and adsorption of *S.mutans* on enamel surfaces.<sup>146</sup>

### **Preparation of Plant Extract**

The collected fresh leaves were washed and air dried at room temperature at shade. After being dried, they were grounded into coarse powders, which were placed in containers and stored at room temperature until the extraction procedure. The alcoholic extract of the ground plant extract prepared by cold maceration by taking 400 g of an individual powder and suspending it in 1000 mL of ethanol (95%) for six to seven days. After completion of four days, the mixture was gravity filtered through Whatman #4 qualitative filter paper. The brownish clear solutions were concentrated under vacuum to yield about 5 g of a brown solid mass.



## MATERIALS AND METHODS

Test tubes with heat resistant rubber cork.

Sterilized Nichrome Wire No:20

Sterile petridishes

Autoclave

Hot air oven

Colony counter

Stereomicroscope

*S.mutans* (MTCC 890 strain)

Brain Heart Infusion broth and Agar media (High Media)

Fluid thioglycolate medium (High Media)

### Bacterial growth conditions

The bacterial strain used in this study were *Streptococcus mutans* MTCC 890, was obtained from (Institute of Microbial Technology, Sector 39-A, Chandigarh). These strains were cultured in Brain Heart Infusion broth under aerobic conditions at 37<sup>0</sup> C. Their identities were reconfirmed on the basis of morphological data using gram staining technique (Fig No – 8) . All organisms were maintained in either a frozen or lyophilized state. For experiments, working cultures were maintained by passage in Brain Heart Infusion Broth.

## COMPOSITION OF MEDIUM

### Brain Heart Infusion Broth

Components	Gm/lit
Calf brain infusion	200
Beef infusion	250
Protease peptone	10
Dextrose	2
Sodium chloride	5
Disodium phosphate	2.5
Final pH	7.4±0.2

### Fluid Thioglycolate Medium

Components	Gm/lit
Pancreatic digest of casein	15
Yeast extract	5
Dextrose	5
Nacl	2.5
L Cystiene	0.5
Sodium thioglycolate	0.5
Resazurin sodium	0.001
Agar	0.75

## **PREPARATION OF THE BRAIN HEART INFUSION AGAR**

About 2.5 gm of BHI medium dissolved in 50 ml of distilled water, heat to boiling to dissolve the medium completely, sterilized by autoclaving at 15lbs pressure (121°C for 15 mts). The medium was poured into the sterile petridishes to get a thickness of 5-6mm. The medium was allow to solidify and petridish was inverted and were dried at 37°C just before inoculation.

## **PREPARATION OF BRAIN HEART INFUSION BROTH**

Dissolve 1.5 gm of medium in 50 ml of distilled water, heat to boiling till the medium is completely dissolved. Pour the medium (10 ml) in cotton plugged glass tubes and sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes.

## **IN VITRO PLAQUE FORMATION ON NICHROME WIRES<sup>106</sup>**

In vitro plaques were grown on no.20 Nichrome wires using a slight modification of methods previously detailed. For plaque growth study, 24-h brain heart infusion cultures (0.2 ml) were used to inoculate into test tubes (18 x 150 mm) containing 10 ml of Fluid Thioglycolate medium supplemented with 5% w/v sucrose and 0.005% w/v sodium carbonate. The Nichrome wires were fixed to rubber stoppered culture

tube closures were transferred daily to fresh medium (approximately 3 to 4 days) until the wire adherent of microorganisms (in-vitro plaque).

All cultures were incubated at 37° C. (Plate No - 26 )

### **PREPARATION OF TEST AND STANDARD SOLUTIONS**

Ethanollic extract of the plant was dissolved in 0.5% v/v Tween 80 and was diluted to final concentrations 5, 10, 25, 50, 75, 100, and 150 mg/ml using distilled water. Commercial antimicrobial agent Chlorhexidine gluconate (0.2% v/v) is used as standard.

### **ANTIPLAQUE EFFICIENCY OF THE EXTRACT AND QUANTIFICATION**

The wire adherent plaques were removed and dipped in the test and standard solutions for five and 10 minutes.

Then the wire adherent plaques were then rinsed twice in 15 ml of distilled water. The plaque was removed from the wires, suspended in 5 ml of sterile 0.85% NaCl, and subjected to five 15-second bursts of ultrasonic oscillation.

Aliquots of the dispersed plaque suspensions were diluted in sterile 0.85% NaCl and spread over petridishes containing brain heart infusion agar. The plates were incubated aerobically at 37°C for 24 hrs. Colonies were counted and compared with that of standard. The results were tabulated and photographs were taken. (Plate – 27 to 40, Table – 17, 18, Fig.9 )

## RESULTS AND DISCUSSION

The dissertation covers the works on Pharmacognostic, preliminary Phytochemical, invitro dental plaque biofilm formation inhibition and mutagenic effect of *W.tinctoria* Family: **Apocynaceae** in an attempt to rationalize its uses as a safe drug of therapeutic importance of plant origin.

**Chapter I: Introduction:** We discussed the importance of medicinal plants □ Plants as sources for medicine □ Medicinal plants in India □ Herbal medicine for healing and health □ Alternative system of medicine including Ayurveda, Siddha, and Unani □ Phytopharmaceuticals □ Contributions of pharmacognosy to modern medicine □ Oral infections and systemic diseases □ Dental Plaque □ Factors influencing plaque formation □ Health hazards due to dental plaque □ Herbal treatment for plaque □ stages of biofilm formation □ Reason for the selection of the plant *W.tinctoria*.

**Chapter II: Review of Literature :** The various literatures available were categorized under Ethnomedical, Pharmacognostical, Phytochemical, Pharmacological, Microbiological screening of whole plant, leaves, flowers, fruits, seed, pod, bark, root bark, and wood and various methods to evaluate in-vitro dental plaque formation have been reviewed.

**Chapter III: Aim and Objective** were planned to study the Pharmacognosy, Phytochemistry, and in-vitro dental plaque biofilm formation inhibition by the leaf extract of *W.tinctoria*. Moreover a simple reliable technique designed to study the mutagenic effect. In addition the above aim was set by considering the following factors

A new hope for the control of this neglected disease.

To provide significant contribution to the chemotherapy of the dental diseases by natural product derived compound in future.

To study the scientific use as tooth ache and odontalgia.

#### **Chapter IV : Section A : Pharmacognostic Studies**

Macroscopical study including taxonomical position □ geographical distribution □ the habit and habitat □ description of leaves, bark, inflorescence, flowers, pods , seeds, and wood □ seasons of flowering □ means of propagation were discussed.

Photograph and line drawings were presented as an establishment of authenticity. (Plate No 1-8, Fig-2)

### **Morphology of the leaf**

Alternate	:	Bipinnate
Size	:	3-6 inches long and 1-2 1/2 inches broad.
Leaflets	:	Short petioled with 6-12, close to each other.
Shape	:	elliptic, lanceolate, obovate to oblong
Margin	:	Slightly serrate
Apex	:	Acute or acuminate
Colour	:	Pale green
Odour	:	No characteristic odour.
Taste	:	Pungent
Texture	:	Smooth

### **Bark**

Pale grey smooth thin bark which is reddish brown in color.

### **Flowers**

Flowers are numerous, bisexual, white color fragrance flowers.

The flowers yield a blue dye.

**Fruit**

Fruits of two, initially green and becomes brown when mature, very long, follicles in pairs, pendulous, cylindrical in nature, and coadhering at their tips.

**Seeds**

Numerous, linear, glabrous, slender, pointed at apex with a fine silky hairs often more than 11/2 inch long.

**Wood**

Uniformly white when first exposed, turning ivory colored with age, even grained, lustrous, smooth.

**Section B**

Deals with the Microscopical studies of the leaf to ascertain the arrangement of tissues (Plate No: 9-16). The T.S. of leaflet through midrib and the powder analysis showed the following features.

**MIDRIB**

Thick wide pot shaped, (1.5 mm vertically, 1.3 mm horizontally)

**ADAXIAL SIDE**

Shallow and wide slight hump, has four or five layers of collenchymas cells, apostamatic, amoeboid in outline, anticlinal walls are thick and wavy.

**ABAXIAL SIDE**



Homogenous parenchyma cells with intracellular spaces, abaxial epidermis is stomatiferous, paracytic type, (60-70  $\mu\text{m}$  long, 30-40  $\mu\text{m}$  wide).

## GROUND TISSUE

Wide circular cells which are **laticifers** or latex secreting cells which are random in distribution without any inclusions.

## VASCULAR BUNDLE

Wide, deep, and U Shaped.

**Xylem** – Closely arranged rows of xylem elements, **five** in each rows, They are angular and thick walled. **Meta xylem** is 40  $\mu\text{m}$  wide.

**Phloem** - Small circular units both outer and inner side of the xylem. (wider)

## LAMINA

220  $\mu\text{m}$  thick, adaxial epidermis which are thick walled, tabular, (20  $\mu\text{m}$  thick). Abaxial epidermis is thick walled **squarish** cells (15  $\mu\text{m}$  thick.)

## MESOPHYLL

Short narrow cylindrical **palisade** cells (50 µm height.) **Calcium oxalate** druses **abundant** under Polarized Light Microscope (50 µm in diameter). **Spongy mesophyll** is five to six layered, spherical or loosely linked with each other forming wide air chambers.

## VENATION

Vein islets are wide, distinct, varying in size and shape. Well developed, vein terminals invariantly forked repeatedly forming **dendroid configuration**.

## PETIOLE

Pot shaped, (5 mm horizontally and 4 mm vertically.) The adaxial lateral twigs are not prominent. Epidermis consists of continuously arranged squarish cells. The ground tissue is totally parenchymatous lacking collenchymas. The vascular system has one wide bowl shaped main strand and two small circular lateral accessory strands. (1.4 µm horizontally and 400 µm thick.) The xylem thick walled. Phloem occurs in discrete strands both on the inner and outer sides of the Xylem.

**Section C** deals with the quantitative microscopy in terms of microscopic and physical parameters. The leaf constant values including vein islet and vein termination number, stomatal number and index, and palisade ratio of ***W.tinctoria*** were given (Table No 3 - 6).

Sl.No:	PARAMETERS	MINIMUM	AVERAGE	MAXIMUM
1.	Vein islet number	6	6.9	9
2.	Vein termination number	7	8.7	10
3.	Stomatal number (LE)	75	79.9	84
4.	Stomatal Index (LE)	17.51	18.64	20.12
5.	Palisade Ratio	5	7	9

The physical parameters were tabulated from Tabulated from Table (7-10)

Parameters	Minimum	Average	Maximum
Total ash	8.41	9.40	10.21
Acid insoluble ash	0.67	0.83	0.98
Water soluble ash	5.59	5.96	6.12
Loss on drying	7.48	7.8	8.3

### Extractive values (individual solvent extraction)

Solvents	Extractive value (%)
Petroleum ether	1.3
Benzene	0.06
Ethyl acetate	0.92
Chloroform	1.89
Ethanol	2.12
Water	5.4

## EXTRACTIVE VALUES (SUCCESSIVE SOLVENTS)

Solvents	Extractive Value (%)
	Leaf
Petroleum ether	1.42
Benzene	0.32
Ethyl acetate	0.62
Chloroform	1.22
Ethanol	2
Water (reflux)	4.01

**Chapter V: Section A** deals with the preliminary phytochemical screening of the powdered leaves and the results were tabulated in (Table No-11).

Constituents present	Constituents absent
Carbohydrates, phytosterols, alkaloids, glycosides, tannins, proteins & amino acids, terpenoids, flavanoids	Saponins, fixed oil, volatile oil gums & mucilage,

### Phytosterols (+)

Carbohydrate(+) Proteins (+) Aminoacids (+)  
Tannins (+)  
Glycosides (+)

Phytosterols (+) Terpenoids (+)  
Carbohydrate (+) Flavanoids (+)  
Glycoside (+)  
Tannins (+)  
Alkaloids (+)

Phytosterols (+)  
Terpenoids (+)

Aqueous Extract

Petroleum Ether Extract

Ethanolic Extract

Benzene Extract

Chloroform Extract

Ethyl Acetate Extract

Phytosterols (+)

Terpenoids (+)

Leaf Powder

Schematic representation of various constituents present in the leaves of *W.tinctoria*.

**Section B** deals with TLC of ethanolic and ethyl acetate extract, co-TLC of isolated  $\beta$  amyrin & HPTLC of the ethanolic and ethyl acetate extracts of leaves of *W.tinctoria*.

TLC was developed using Toluene: Acetonitrile (7:3) as solvent system on Silicagel 60F<sub>254</sub> as adsorbent. Three spots in Ethanolic extract with R<sub>f</sub> values 0.3, 0.55, and 0.8 (pink, pale green, dark green) and two spots in ethyl acetate extract with R<sub>f</sub> values (0.2 and 0.7) were observed. (Table -13, Plate - 17).

HPTLC profile of ethanolic and ethyl acetate extract of ***W.tinctoria*** leaves using Toluene: Acetonitrile (7:3) showed 6 and 7 peaks.

(Table -14, 15, Fig. 5,6 )

**Section C:** deals with the isolation, identification, and TLC of the isolated  $\beta$  amyrin from the petroleum ether extract of leaves of ***W.tinctoria***. The mixed melting point readings and R<sub>f</sub> value matches with the standard  $\beta$  amyrin. So the isolated compound may be  $\beta$  amyrin (Plate No - 18 ).

In **Chapter VI: Section A** deals with the mutagenic effect of the leaves of ***W.tinctoria***. In this method, we discussed about the need for performing the Eye and Wing Spot assay, Description about *D.melanogaster*, Life cycle and reproduction, Model organism in genetics, and its similarity to humans.

The II<sup>nd</sup> stage larvae were selected for experiment. Formaldehyde (0.5% v/v) selected as standard chemical mutagen. Ethanolic extract in the concentrations (5, 10, 25, 50 µg/ml) range were employed. After eclosion of the exposed flies from larvae stage, the Wing Spot Assay was performed. In this morphological changes including eye color, wing hair, wing spot, wing shape, changes in the wing length and width, abdomen length, and total body length were observed. Standard mutagen (0.5% v/v) produced visual mutations (eye color become pink from brown) and additionally there was noticeable changes in the wing length and width, but remaining factors unchanged when compared to normal flies. The results are tabulated (Table – 16). Values are represented as  $\pm$ S.E.M. values. From the results it was observed that the ethanolic extract at all concentrations ((5, 10, 25, 50 µg/ml) showed no significant morphological changes in the ***D.melanogaster on comparing Formaldehyde exposed flies***. This

preliminary assay suggests its antimutagenic effect on *D.melanogaster*. This method needs further investigation to carry out *invivo* tests in germ cells, as well as in somatic cells in order to assess the effects in genetic level.

**Section B** deals with the in-vitro dental plaque biofilm formation inhibitory activity of ethanolic extract of ***W.tinctoria***. In this we discussed about the role of dental plaque in caries formation and periodontal diseases. Confocal laser scanning microscopy showed that the structure of gingival plaque has open architecture, presence of dead and live stains in the biofilm. We discussed about the stages of plaque formation, factors responsible for biofilm formation and maturation, and role of biofilm in the development of plaque. We discussed the structure and scientific classification of *S.mutans* and its participation in the formation of dental plaque. *S.mutans* is the primary colonizer bacterium which has a major role in tooth decay. *S.mutans* converts the sucrose in to lactic acid and creates an acidic environment and mineralizes the tooth leading to caries formation and tooth decay. We discussed about the antimicrobial agents for plaque and their side effects. So we reviewed the need for newer dental plaque inhibitors which are plant based. Medicinal plants usually have multiple Phyto-constituents to act synergistically act with various targets, have better bio-availability and efficacy. We are interested to study this plant



extract to provide a scientific validation for the traditional use of tooth ache and odontalgia.

Ethnomedical claims revealed that the leaves are chewed for the relief of tooth ache and the review of literature revealed the presence of tryptanthrin, and its bactericidal effect was already proved. So we planned to study the extract of the leaves of the plant against invitro plaque inhibition formed by primary colonizer bacterium *S.mutans*.

Methods for evaluation of plaque using artificial dental biofilm model, saliva conditioned flow cell model, evaluation using quartz crystal microbalance, Zurich biofilm model has been reviewed. But these methods are expensive cum complicated, so we designed a method for screening plant material for this activity which is adaptable to natural and synthetic materials, simple, rapid, and reliable technique. In vitro plaques were formed on Nichrome wires no:20 by the slight modification of the methods which are previously described. According to those methods, the wires were inserted in the complex medium with 5% v/v sucrose and sodium carbonate. The preparation of the above mentioned medium was laborious complex, and expensive, so we

substituted fluid thioglycolate medium instead of complex medium as stated above. Initially we standardized the procedure for deposition of plaque formed by the *S.mutans* over Nichrome wires in fluid thioglycolate medium supplemented with sucrose and sodium carbonate. The plaque deposition was uniform and without any contaminants. So we further proceeded our work with the above stated procedure. The ethanolic extracts of the plant was dissolved in tween 80 (0.5% v/v), tween 80 was selected since it is proved to have nonbactericidal effect on *S.mutans*.

It was observed that the extracts inhibited the growth of *S.mutans* in dose dependent manner. The no of colonies formed on 5 min and 10 min exposure to the test, std, control were tabulated and the photographs were presented. (Table no - 17, 18, Plate - 25 to 40, Fig.:9). Exposure to 100 and 150 mg of extract to five and ten minutes showed complete inhibition of the organism. So it was obvious that plaque inhibition prevention by the ethanolic extract ranges between 100 to 150 mg/ml concentrations.

The increased resistance of biofilms to antimicrobial reagents are subjected to much research and debate. In this investigation the ethanolic extract of leaves of ***W.tinctoria*** showed significant antibiofilm activity, however, it is only a model but the actual activity on dental

plaque reduction has to be evaluated in further clinical trials. Cells can become resistant to mutation affecting the drug target. The safety of the drug on the cell was further evaluated by its mutagenic effect on *D.melanogaster*.

# CONCLUSION

This dissertation covers the Pharmacognostic parameters of the leaves of *Wrightia tinctoria* belonging to the Family **Apocynaceae** such as macroscopical, microscopical including powder analysis and physical standards like ash value, extractive values etc have been studied and presented.

The tissue arrangement in the leaf was studied and presented. Specific microscopical characters of the leaf were as follows: Ground tissue contains wide circular cells which are **latricifers** or latex secreting cells which are random in distribution without any inclusions. In the venation pattern vein terminals are invariantly forked repeatedly forming **dendroid configuration**. **Calcium oxalate** druses **abundant** under Polarized Light Microscope (50  $\mu\text{m}$  in diameter).

The preliminary phytochemical studies in leaf powder revealed the presence of carbohydrate, phytosterols, alkaloids, glycosides, protein and amino acids, terpenoids, and flavanoids.

Isolation and identification of  $\beta$  amyryn from the petroleum ether extract.

HPTLC of the ethanolic and ethyl acetate extracts were studied and presented.

**Antimutagenic Study:** A simple, economical, reliable laboratory scale Wing Spot Assay on *D.melanogaster* to assess the mutagenic effect was standardized and presented. In the present study it was confirmed that the ethanolic extract is found to be nonmutagenic

(5–50 µg/ml) on *D.melanogaster*. It need further advanced genotoxic experiments in animal model using SMART assay. (Somatic mutation and Recombination Test Assay).

**Invitro Dental Plaque Biofilm Inhibitory Activity:** The results of the current investigation revealed that the biofilm (Plaque) formation by the *S.mutans* was inhibited by the ethanolic extract was concentration dependent. There was nil growth of *S.mutans* observed at 100 and 150 mg/ml concentration.

So we were also able to demonstrate significant invitro dental plaque biofilm inhibitory activity was exhibited by the ethanolic extract of the leaves of ***W.tinctoria*** against Primary Colonizer Bacterium *S.mutans*. Moreover this study provides the scientific basis for the Ethnomedical use of this plant as dental analgesic.

***The above study require further investigation for the exact mechanism of action, to develop a safe herbal based mouth rinse formulation for the prevention of Dental Plaque and for the maintenance of oral hygiene.***

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